Recombinant Epstein-Barr Virus Proteins and Antibody-selected Peptides: Reagents for Serodiagnosis and Vaccination

A dissertation submitted for the degree of Ph.D.

by

Olivia Flynn B.Sc.

Under the supervision of Dr Dermot Walls

January 1999

School of Biotechnology, Dublin City University, Dublin 9, Ireland
Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: ____________________________  I.D. No. 94971145
Olivia Flynn

Date: _______________________________
To my Dearest Orla,
Forever in my Heart.
Acknowledgments

I would like to thank my parents for all their love, support, continued encouragement, and for believing in me, especially when I didn’t. Also a big thanks to Monica, Kevin, Joan and Sorcha for putting up with me when the nerves were fraying. Thanks to my grandparents, their perseverance in difficult times was an inspiration.

Thanks to Úna and Sinéad who have shared the good times and the bad all in the spirit of friendship, and for the hours of listening without complaint. To Finola, Daragh, Trish, Jen, Stella and Dee, for the friendship, encouragement and all the laughs, I will always be grateful.

I wish to thank my supervisor Dr Dermot Walls for the opportunity to undertake a Ph.D. in his laboratory and for his continued advice and support. A special thanks to Mel for her work on the large-scale protein purification and her inimitable wit and wisdom.

Thanks to all in Dr Susan McDonnell’s, Prof John Dalton’s and Prof Richard O’Kennedy’s laboratories past and present, especially Maria who I could always rely on whatever the situation, and to Carol, Barbara and Sinéad for the advice in the early days. Thanks also to the staff and postgrads of the Biotechnology Department who made DCU a pleasant place to work.

To Joanne, Pam M., Mel, Brendan and Pam P., thanks for the unforgettable coffee breaks and for making AG24 the best lab to work in, with the perfect balance of business and pleasure.

For all the technical assistance in producing this thesis, thanks to Sinéad McGrath, James’ Hospital, Úna Cusack, Daragh Byrne and Oonagh Dowling.

iv
Thanks Gar for the fun trips to the cinema, Martina for the sumptuous meals and Ben our reliable Mr Fix-it, and to each of you for the much appreciated trips to Laois and to DCU

Thanks John for your understanding and patience, Renée the quintessential yoga guru, and Pepé for always bringing a smile to my face
Recombinant Epstein-Barr Virus Proteins and Antibody-selected Peptides: Reagents for Serodiagnosis and Vaccination.

Olivia Flynn

Abstract
The Epstein-Barr virus (EBV) is a ubiquitous human oncogenic herpesvirus which causes infectious mononucleosis (IM) and contributes to the development of many important cancers in man. The objectives of this thesis were twofold:

1. the development of recombinant EBV antigens as reagents for the serodiagnosis of EBV-related disease,
2. the identification of peptides that bind EBV neutralizing antibody using phage display technology

Serology testing is of paramount importance in the diagnosis of EBV-related diseases, with viral antigen for these tests usually being derived from cell culture. The production of the appropriate recombinant EBV antigens in a prokaryotic system would eliminate many of the disadvantages associated with cell culture-derived EBV antigens, i.e. reproducibility, cross-reactivity and expense. The coding sequences for several targeted EBV proteins were cloned into an E. coli expression vector (Thiofusion system). In this system recombinant proteins were fused to E. coli thioredoxin thus offering the advantages of stability, solubility, inducibility and ease of purification. Three EBV antigens, p18VCA, EA-D and EBNA1, were chosen for expression and purification. These recombinant antigens were assayed with sera that had been previously partially characterised for antibodies to EBV. A cut-off point was determined by comparison of ELISA and Western blot results. The antibody profiles of each serotype, i.e. infectious mononucleosis-positive sera, sera from normal healthy individuals, VCA+- sera and EBNA+- sera, agreed with those determined using cell culture-derived antigen in other assay systems. Some potential cross reactivity with CMV was noted. These recombinant proteins may be of use as immunodiagnostic reagents for the diagnosis of EBV-related diseases.

The EBV envelope glycoprotein gp350 mediates virus adsorption and penetration to its host cell and is the principal candidate subunit vaccine. The monoclonal antibody 72A1 is known to neutralise EBV through interaction with gp350. The aim was to identify peptides that bind to 72A1 using phage display technology. Using this antibody, peptide sequences were selected which showed specific similarities in amino acid content and arrangement, however it was not possible to show specific interaction between the selected clones and 72A1 by other immunological methods. As only one group of clones from the set of 15mer sequences bears similarity to the native gp350 primary amino acid sequence, it is most likely the epitope is conformational, or that all other selected peptides are mimotopes of a linear epitope.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adenosine</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorbance</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>ARL</td>
<td>AIDS Related Lymphoma</td>
</tr>
<tr>
<td>AS</td>
<td>Ammonium Sulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri-phosphate</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt’s Lymphoma</td>
</tr>
<tr>
<td>BPTI</td>
<td>Bovine Pancreatic Trypsin Inhibitor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cytidine</td>
</tr>
<tr>
<td>CBD</td>
<td>Chitin Binding Domain</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFS</td>
<td>Chronic Fatigue Syndrome</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf Intestine Alkaline Phosphatase</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CP</td>
<td>Constrained Peptide</td>
</tr>
<tr>
<td>CR</td>
<td>Complement Receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’- Diaminobenzidine</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxy-Adenosine Tri-phosphate</td>
</tr>
<tr>
<td>DBP</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>DCL</td>
<td>Disulphide-Close Loop</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EA-D</td>
<td>Early Antigen- Diffuse</td>
</tr>
<tr>
<td>EA-R</td>
<td>Early Antigen- Restricted</td>
</tr>
<tr>
<td>EBER</td>
<td>Epstein-Barr virus Encoded RNA</td>
</tr>
<tr>
<td>EBNA</td>
<td>Epstein-Barr virus Nuclear Antigen</td>
</tr>
<tr>
<td>EBL</td>
<td>Endemic Burkitt’s Lymphoma</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetracetic acid</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immuno-Assay</td>
</tr>
<tr>
<td>EK</td>
<td>Enterokinase</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FHD</td>
<td>Familial Hodgkin’s Disease</td>
</tr>
<tr>
<td>G</td>
<td>Guanosine</td>
</tr>
<tr>
<td>GMT</td>
<td>Geometric Mean Titer</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HBsAg</td>
<td>Hepatitis B surface Antigen</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalovirus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin’s Lymphoma</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HNE</td>
<td>Human Neutrophil Elastase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HT</td>
<td>Heat Treatment</td>
</tr>
<tr>
<td>HVS</td>
<td>Herpes Virus Saimiri</td>
</tr>
<tr>
<td>IB</td>
<td>Inclusion Body</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence Assay</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interlukin</td>
</tr>
<tr>
<td>IM</td>
<td>Infectious Mononucleosis</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-Thiogalactopyranoside</td>
</tr>
<tr>
<td>ISCOMS</td>
<td>Immunostimulatory Complexes</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese Encephalitis Virus</td>
</tr>
<tr>
<td>kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luna-Bertram broth</td>
</tr>
<tr>
<td>LCL</td>
<td>Lymphoblastoid Cell Line</td>
</tr>
<tr>
<td>LMP</td>
<td>Latent Membrane Protein</td>
</tr>
<tr>
<td>LP</td>
<td>Latent Protein</td>
</tr>
<tr>
<td>LPD</td>
<td>Lymphoproliferative Disorder</td>
</tr>
<tr>
<td>MA</td>
<td>Membrane Antigen</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose Binding Protein</td>
</tr>
<tr>
<td>MDBP</td>
<td>Major DNA Binding Protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>NHS</td>
<td>3- Sulfo-N-hydroxysuccinamide ester</td>
</tr>
<tr>
<td>NIP</td>
<td>4-Hydroxy-3-iodo-5-nitrophenylacetic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NPC</td>
<td>Nasopharyngeal Carcinoma</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonylphenoxypolyethoxy ethanol - 40</td>
</tr>
<tr>
<td>NT</td>
<td>Neutralisation Assay</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OHL</td>
<td>Oral Hairy Leukoplakia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>OPD</td>
<td>O-Phenylenediamine</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>ori</td>
<td>Origin of replication</td>
</tr>
<tr>
<td>OS</td>
<td>Osmotic Shock</td>
</tr>
<tr>
<td>P</td>
<td>Pellet</td>
</tr>
<tr>
<td>p</td>
<td>Plasmid</td>
</tr>
<tr>
<td>p</td>
<td>Protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen Activator Inhibitor</td>
</tr>
<tr>
<td>PAO</td>
<td>Phenylarsine Oxide</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCAb</td>
<td>Polyclonal Antibody</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-Nitrophenyl phosphate</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PTLD</td>
<td>Post-transplant Lymphoproliferative Disorder</td>
</tr>
<tr>
<td>PTX</td>
<td>Bordatella Pertussis Toxin</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination Activating Genes</td>
</tr>
<tr>
<td>RBP-Jk</td>
<td>Recombination signal Binding Protein - Jk</td>
</tr>
<tr>
<td>rec</td>
<td>Recombinant</td>
</tr>
<tr>
<td>RF</td>
<td>Replicative Form</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPL</td>
<td>Random Peptide Library</td>
</tr>
<tr>
<td>RS</td>
<td>Reed-Sternberg</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory Syncitial Virus</td>
</tr>
<tr>
<td>S</td>
<td>Supernatant</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>T</td>
<td>Thymidine</td>
</tr>
<tr>
<td>Ta</td>
<td>Annealing temperature</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffer saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffer saline plus Tween 20</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N' - Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine Kinase</td>
</tr>
<tr>
<td>TNT</td>
<td>Tris, NaCl, Tween 20</td>
</tr>
<tr>
<td>TR</td>
<td>Terminal Repeat</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>UP</td>
<td>Unstructured Peptide</td>
</tr>
<tr>
<td>UP H₂O</td>
<td>Ultra pure water</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCA</td>
<td>Viral Capsid Antigen</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella Zoster Virus</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-chloro-3-indoyl-β-D-galactoside</td>
</tr>
<tr>
<td>XPL</td>
<td>X-linked Lymphoproliferative Disorder</td>
</tr>
<tr>
<td>Z</td>
<td>ZEBRA</td>
</tr>
<tr>
<td>ZEBRA</td>
<td>BamH1 Z Epstein-Barr virus Replication Activator</td>
</tr>
<tr>
<td>Units</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micromole</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>g</td>
<td>g force</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mM</td>
<td>Milliamps</td>
</tr>
<tr>
<td>mn</td>
<td>Minute</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Mlimolar</td>
</tr>
<tr>
<td>mol</td>
<td>Moles</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>pmole</td>
<td>Picomole</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RU</td>
<td>Response units</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>TU</td>
<td>Transducing unit</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
</tbody>
</table>
Figures

Figure 1  BamHI-restriction map of the EBV genome
Figure 2  Model of EBV-host interaction during primary infection and persistence
Figure 3  Schematic representation of EBNA 2
Figure 4  Schematic representation of EBNA 1
Figure 5  Antibody responses during EBV-induced infectious mononucleosis
Figure 6  Schematic representation of the pTrxFus plasmid
Figure 7  Schematic representation of the adhesion zones
Figure 8  Filamentous bacteriophage displaying peptides fused to pIII and pVIII
Figure 9  Classification of phage display vectors
Figure 10 Flow diagram of the biopanning procedure
Figure 12 Schematic representation of the EBNA1 protein
Figure 13 Amplification of 829 bases of the BKRF1 ORF by PCR
Figure 14 Amplification of the BFRF3 ORF by PCR
Figure 15 Amplification of the BMRF1 ORF by PCR
Figure 16 Amplification of the BARF0 ORF by PCR
Figure 17 Schematic representation of the pTrxFus plasmid
Figure 18 The multiple cloning site of pTrxFus
Figure 19 Schematic maps of each recombinant plasmid
Figure 20 Agarose gel showing each of the plasmids with the insert ligated, uncut
Figure 21 DNA agarose gel analysis of BamH1 digest of each recombinant plasmid
Figure 22 DNA agarose gel analysis of Sma1 digest of each recombinant plasmid
Figure 23 DNA agarose gel analysis of Rsa1 digest of pTrxpl8VCA
Figure 24 Expression of soluble recombinant rVCA p18 in E coli
Figure 25 Soluble fraction of rBARF0
Figure 26 Effects of increasing somcation time without freeze-thaw, on Trx
Figure 27 Effects of increasing somcation time without freeze-thaw, on rEBNA1
Figure 28 Crude preparations and the soluble fraction, after somcation, of both Trx and rEA
Figure 29 Purification of Trx and rEA by osmotic shock
Figure 30 Purification of rVCAp18 by osmotic shock
Purification of Trx and rVCAp18 by heat treatment at 80°C.

Ammonium sulphate (AS) precipitation of rVCAp18 - 1st cut.

Ammonium Sulphate precipitation of rEBNA1 - 1st and 2nd cut.

Purification of Trx using PAO agarose.

Purification of rEBNA1 using PAO agarose.

Purification of rVCAp18 and rEA using PAO agarose.

SDS-PAGE gel of protein standards and rVCAp18 and rEBNA1 for densitometry.

SDS-PAGE gel of protein standards and rEBNA1 and rEA for densitometry.

Photograph of the Monosticon DRI-DOT test cards after serum was added.

Western blot analysis of Trx and rVCAp18 after sonication, with six IM+ sera.

Detection of rVCAp18 by western blotting.

Detection of rVCAp18 by western blotting.

Detection of IgG Ab response to the 3 recombinant EBV antigens.

Detection of IgM Ab response to the 3 recombinant EBV antigens.

Western blot analysis of purified recombinant proteins with CMV+ and IM+ sera.

Flow diagram of the biopanning procedure for the 15 mer phage library.

Flow diagram of the biopanning procedure for the 7 mer phage library.

Flow diagram of the biopanning procedure for the 7 mer constrained phage library and the 12 mer library.

Analysis of phage template DNA prior to sequencing.

Analysis of phage template DNA prior to sequencing.

Photograph of the sequencing autoradiograph, showing sequences from the 15mer library.

Alignment of the sequences selected from the 15 mer peptide library using 72A1, according to amino acid class.

Alignment of the sequences selected from the 7 mer peptide library using 72A1, according to amino acid class.
Figure 55  Alignment of the sequences selected from the 7 mer constrained peptide library using 72A1, according to amino acid class
Figure 56  Alignment of the sequences selected from the 15 mer peptide library using F2 1, according to amino acid class
Figure 57  The primary amino acid sequence of gp350 colour coded for amino acid class
Figure 58  A 7 5% SDS PAGE gel of purified gp350
Figure 59  Western blot analysis of purified gp350
Figure 60  Western blot analysis of gp350, glycosylated and deglycosylated, with 72A1, F29 167 and MDP 61 1 4 polyclonal antibody preparation
Figure 61A  Plaque lift of pfu s from the eluate of the third round of panning with the 7mer constrained library using the alkaline phosphatase anti-mouse conjugate and BCIP/NBT substrate
Figure 61B  The controls used for the plaque lift
Figure 62A  Plaque lift of pfu s from the eluate of the third round of panning with the 7mer constrained library using the peroxidase anti-mouse conjugate and a chemiluminescent substrate
Figure 62B  The controls used for the plaque lift
Figure 63  DOT blot format for detection of 72A1 - specific peptides on phage particles selected during biopanning
Figure 64  Auto-radiograph of DOT Blot assay carried out with phage from the 7mer constrained library
Figure 65  Sensorgram of immobilisation of 72A1 on the sensor chip
Figure 66  Sensorgram of the binding of gp350
Figure 67  Possible ELISA formats for detection of 72A1 - specific peptides on phage particles selected during biopanning
Tables

Table 1: International nomenclature of herpesviruses.
Table 2: EBV-Associated Malignancies.
Table 3: Patterns of EBV latent gene expression
Table 4: Serological patterns of EBV antibodies in patients with EBV-associated syndromes
Table 5: Polymerase Chain Reaction (PCR), standard program.
Table 6: Restriction Analysis, predicted fragment sizes.
Table 7A: Protein Concentration of each Recombinant Protein.
Table 7B: Approximate yield of each of the recombinant proteins
Table 8: Monosticon DRI-DOT results from a selection of IM - positive and Normal Sera.
Table 9: Predicted IgG and IgM Ab responses of normal (EBV+) and IM+ sera
Table 10: Details of sample preparation for ELISA assay.
Table 11: The ELISA assays which were carried out.
Table 12: ELISA vs Western Blot anti-IgG results of 9 IM+ and 10 normal sera.
Table 13: ELISA vs Western Blot anti-IgM results of 9 IM+ and 10 normal sera.
Table 14: IgG and IgM responses of normal sera to each of the recombinant antigens.
Table 15: IgG and IgM responses of IM+ sera to each of the recombinant antigens.
Table 16: IgG response of EBNA-/+ sera to rEBNA1.
Table 17: IgM response of VCA-/+ , CMV+ and EBV+ sera to rVCAp18.
Table 18: Phage display libraries
Table 19: Anti-EBV monoclonal antibodies
Table 20A: Titering results for the 7mer library when panned with 72A1 and F29.167.
Table 20B: Titering results for the 7mer constrained library when panned with 72A1.
Table 21: Amino acid sequences displayed on phage clones selected with the anti-gp350 virus-neutralising Mab 72A1 from the 15mer library.
Table 22: Amino acid sequences displayed on phage clones selected with the anti-gp350 virus-neutralising Mab 72A1 from the 7mer library.
Table 23  Amino acid sequences displayed on phage clones selected with the anti-gp350 virus-neutralising Mab 72A1 from the 7mer constrained library
Table 24  Amino acid sequences displayed on phage clones selected with the anti-gp350 virus-neutralising Mab F21 from the 15mer library
Table 25  Amino acid sequences displayed on clones selected with 72A1 from the primary libraries and from various stages throughout the biopanning procedure
Table 26  The Controls used for the Colony/Plaque Lifts
Table 27  Titration results of a selection of phage purified from the eluate from the third round of panning of the 7mer library with 72A1

Graphs

Figure A  Anti-p18VCA IgM Responses of VCA-/+ , Normal and IM+ Sera
Figure B  Anti-p18VCA IgG Responses of Normal Sera and IM+ Sera
Figure C  Anti-EBNA1 IgG Responses of EBNA-/+ , Normal and IM+ Sera
Figure D  Anti-EBNA1 IgM Responses of Normal Sera and IM+ Sera
Figure E  Anti-EA IgG Responses of Normal Sera and IM+ Sera
Figure F  Anti-EA IgM Responses of Normal Sera and IM+ Sera
Figure G  Anti-p18VCA/EBNA1/EA-D IgG Responses of Normal Sera
Figure H  Anti-p18VCA/EBNA1/EA-D IgM Responses of Normal Sera
Figure I  Anti-p18VCA/EBNA1/EA-D IgG Responses of IM+ Sera
Figure J  Anti-p18VCA/EBNA1/EA-D IgM Responses of IM+ Sera
Figure K  Anti-p18VCA IgM Responses of EBV+ Sera and CMV+ Sera
Table of Contents

Declaration  ii
Dedication  iii
Acknowledgments  iv
Abstract  vi
Abbreviations  vii
Units  xii
Figures  xiii
Tables  xvi
Graphs  xvii
Table of contents  xviii

1. Introduction  1

1.1 Historical Aspects of Epstein-Barr Virus  2
   1.2.1 EBV Morphology  2
   1.2.2 Genome Structure  4
   1.2.3 Variant EBV Strains  4

1.3.1 Biology of EBV Infection  7
1.3.2 EBV Latent Infection  8
   1.3.2.1 Types of Latency  10
   1.3.2.2 EBER1 and 2  11
   1.3.2.3 EBV Nuclear Antigen (EBNA)  12
   1.3.2.4 EBNA2  12
   1.3.2.5 EBNA3A, 3B and 3C  13
   1.3.2.6 EBNA1  14
   1.3.2.7 LMP1  16
   1.3.2.8 LMP2A and 2B  17
   1.3.2.9 BARFO  17
133 EBV Lytic Infection
   1331 Immediate Early Genes 21
   1332 Early Genes 21
   1333 Late Genes 22

14 EBV and Disease 24
   141 Self-limiting Disease - Infectious Mononucleosis 24
   142 Malignant Disease 26
      1421 Burkitt's Lymphoma 26
      1422 Nasopharyngeal Carcinoma 30
      1423 Hodgkins Disease 32
      1424 AIDS-Related EBV Infection 34
      1425 Posttransplant Lymphoproliferative Disease and EBV 36
      1426 Non-Hodgkin's lymphomas and other Malignancies 37

15 Immunological aspects of EBV-related Disease 38
   151 Serology of EBV Infection 38
   152 Historical Aspects of Serodiagnosis 40
   153 Comparison of Commercially Available Diagnostic Kits 41
   154 Developments in Diagnostics 42

16 Vaccine Development 45
   161 EBV Vaccine Candidate - gp350/220 45
   162 Neutralising Antibodies to EBV 47
   163 The Cytotoxic T Cell Response to EBV 48
   164 A Model of EBV Infection 49
   165 Vaccinia Virus - a Vaccine Vector 50
   166 Adenovirus - a Vaccine Vector 51
   167 Varicella-Zoster - a Vaccine Vector 52
   168 Subunit Vaccines 52
   169 Vaccines Based on Peptide Epitopes for CTLs 53
2. Materials and Methods

2.1 Biological materials

2.1.1 Antibodies

2.1.2 Serum Samples

2.1.3 Oligonucleotides

2.1.4 Commercial Kits and Enzymes

2.2 Chemical Materials

2.3 DNA Manipulation

2.3.1 Storage of DNA Samples

2.3.2 Equilibration of Phenol

2.3.3 Phenol/Chloroform Extraction

2.3.4 Sodium acetate/Ethanol Precipitation of DNA

2.3.5 Ammonium sulphate/Isopropanol Precipitation of DNA

2.3.6 Spectrophotometric Quantification of DNA

2.3.7 Agarose Gel Electrophoresis of DNA

2.3.8 Isolation of DNA from Agarose Gels
2.3.9 QIAEX II Agarose Gel Extraction Protocol  
2.3.10 Decontamination of Ethidium Bromide Solutions  
2.3.11 Restriction Digestion of DNA  
2.3.12 Dephosphorylation of Linearised Plasmid DNA  
2.3.13 Ligation of DNA Molecules  
2.3.14 Small-scale Preparation of Plasmid DNA  
2.3.15 Large-scale Preparation of Plasmid DNA  
2.3.16 Polymerase Chain Reaction Protocol (PCR)  

2.4 Thiofusion Expression System (Invitrogen)  
2.4.1 Bacterial Strains  
2.4.2 Preparation of Chemically Competent Cells using Calcium chloride  
2.4.3 Transformation of Competent Cells  
2.4.4 Expression and Analysis of Transformants  
2.4.5 Induction of Positive Clones  
2.4.6 SDS Polyacrylamide Gel Electrophoresis (PAGE)  
2.4.7 Purification by Osmotic Shock  
2.4.8 Purification by Heat Treatment  
2.4.9 Ammonium sulphate Precipitation of Recombinant Antigens  
2.4.10 Western Blot Analysis of Recombinant antigens  
2.4.11 Protein Purification with PAO Agarose  
2.4.12 ELISA Analysis of Recombinant Antigens with Human Serum  

2.5 Phage Display Libraries (NEB)  
2.5.1 ER2537 E.coli Strain Maintenance  
2.5.2 Phage Titering  
2.5.3 Biopanning Procedure A  
2.5.4 Plaque Amplification  
2.5.5 Rapid Purification of Sequencing Template  
2.5.6 Biopanning Procedure B  
2.5.7 Preparation of Phage for Assay by ELISA and DOT Blot
2.6 Protocols for the 15mer Phage Library (Conley)

2.6.1 Bacterial Strains

2.6.2 Preparation of Terrific Broth Cultures

2.6.3 Titering of Plaque Forming Units (pfu)

2.6.4 Titering of Transducing Units (TU)

2.6.5 Biopanning

2.6.6 Small-scale Propagation and Processing of Phage

2.6.7 Preparation of Sequencing Template

2.6.8 Immunological Screening of Plaques and Colonies

2.7 Sequencing (T7 sequencing kit, Pharmacia Biotech)

2.7.1 Sequencing Reactions

2.7.2 Preparation of Sequencing Apparatus

2.7.3 Casting the Sequencing Gel

2.7.4 Drying and Developing

3. Expression, purification and analysis of recombinant EBV antigens

3.1 Introduction

3.2 Sub-cloning of Antigen coding DNA sequence

3.2.1 Polymerase Chain Reaction (PCR)

3.2.1.1 EBNA1

3.2.1.2 p18VCA

3.2.1.3 EarlyAntigen-D

3.2.1.4 BARF0

3.2.2 Ligation and Transformation

3.2.3 Restriction Analysis
3 3 Expression of Recombinant Protein
   3 3 1 Growth and Induction 129
   3 3 2 Purification of Recombinant Proteins 134
      3 3 2 1 Osmotic Shock 134
      3 3 2 2 Heat Treatment 135
      3 3 2 3 Ammonium Sulphate Precipitation 137
      3 3 2 4 Phenylarsine Oxide (PAO) Agarose 139
      3 3 2 5 Protein Assay and Densitometry 142

3 4 Antigenicity of rProteins 145
   3 4 1 Serum Characterisation 145
      3 4 1 1 Infectious Mononucleosis Sera 145
      3 4 1 2 VCA Positive and Negative Sera 146
      3 4 1 3 EBNA Positive and Negative Sera 146
      3 4 1 4 EBV Positive Sera and CMV Positive Sera 146
      3 4 1 5 Heterophile Antibody Test 147
   3 4 2 Western Blotting with IM Sera and Normal Sera 150
      3 4 2 1 rVCAp18 150
      3 4 2 2 Blotting after Purification using PAO agarose 152
   3 4 3 ELISA using Recombinant EBV Antigens 155
   3 4 4 Establishment of an ELISA Reference Value 164
   3 4 5 Cross-reactivity Testing 171

3 5 Discussion 173
   3 5 1 Purification of the Fusion Proteins 173
   3 5 2 Loss of Expression of rBARF0 175
   3 5 3 Background ELISA Signal 175
   3 5 4 Reference Point for Evaluation of ELISA Data 177
   3 5 5 Antibody Profiles using the Recombinant EBV Antigens 178
   3 5 6 Cross-reactivity with Sera from CMV-positive Individuals 181
   3 5 7 Expression of other recombinant EBV antigens 182
   3 5 8 Future Recommendations 184
4. Identification of anti-gp350 MAb-binding peptides

using phage display. 185

4.1 Introduction 186

4.1.1 The Random Phage Peptide Libraries (RPLs) 187

4.1.2 The Anti-EBV Monoclonal Antibodies 188

4.2 Results 189

4.2.1 Biopanning of the RPLs 189

4.2.2 Phage Titration 193

4.2.3 Sequencing of Selected Phage Clones 194

4.2.3.1 Comparison of Sequenced Clones 203

4.2.3.2 Sequence alignment according to amino acid class 207

4.2.4 Deglycosylation of gp350 and Western Blotting 210

4.2.5 Screening of Selected Phage Clones 214

4.2.5.1 Colony Lifts and Plaque Lifts 214

4.2.5.2 DOT Blotting 218

4.2.5.3 BIAcore Analysis 220

4.2.5.4 ELISA Analysis 222

4.3 Discussion 225

4.3.1 Epitope mapping of gp350 and the neutralising MAb 72A1 225

4.3.1 Sequence Comparisons 225

4.3.2 Methods of Panning 227

4.3.3 Methods of Screening 229

4.3.4 Screening Different Libraries 230

4.3.5 Phage Display Technology 232

5. Conclusions 234

5.1 Expression of recombinant EBV antigens in E. coli 235

5.2 Identification of anti-gp350 MAb-binding peptides 236

6. Bibliography 237
Appendix A

| I  | Solutions for DNA work                     | a          |
|    | 1 General solutions                        | a          |
|    | II Solutions for mini- and maxi- preparation of plasmid DNA | b          |
| I  | Media and Solutions for Thiofusion kit      | b          |
| II | Solutions for SDS PAGE                     | e          |
| III| Solutions for Western Blotting             | f          |
| IV | Solutions for protein purification with PAO resin | g          |
| V  | Solutions for ELISA with recombinant EBV antigens | h          |
| VI | Media and Solutions for peptide libraries (NEB) | h          |
| VII| Media and Reagents for 15mer library        | j          |
| VIII| Reagents for Sequencing                     | m          |
Chapter 1

Introduction to the Epstein-Barr Virus
1.1 Historical Aspects of Epstein-Barr Virus

Epstein-Barr virus (EBV) was discovered during the course of attempts to learn the cause of a lymphoma that was the most common tumour affecting children in certain parts of East Africa. Dennis Burkitt who described the clinical syndrome suggested that this lymphoma might be due to a virus (Burkitt, 1962). Virus particles morphologically similar to the herpesvirus group were found in thin sections of Burkitt’s lymphoma (BL) cell lines (Epstein et al., 1964), in a fraction of cells in lymphoid lines established from patients with various malignancies, from patients with infectious mononucleosis (IM) and from apparently normal individuals (Miller, 1971). On the basis of further studies it was determined that EBV was the cause of IM, since antibodies to the antigens in BL cell lines were absent before mononucleosis and appeared after the disease (Henle et al., 1968). This herpes virus is also consistently detected in nasopharyngeal carcinoma (NPC). (Miller, 1990).

1.2.1 EBV Morphology

Herpesviruses are a family of DNA viruses found commonly in both animals and humans. The known herpesviruses have a common virion architecture, and are primarily classified on the basis of morphology, ie. a core containing a large linear double-stranded DNA genome, an icosahedral capsid, an amorphous tegument and a lipid envelope with viral glycoprotein spikes on it’s surface. The viral particles have a diameter of about 120 to 200 nm and contain 25-35 virus-encoded proteins and host-specific phospholipids derived from the nuclear membrane. The DNA is wrapped around a DNA-associated spindle-shaped protein consisting of fibrils attached to the inside of the capsid. The capsid is surrounded by an amorphous material, tegument, composed of globular proteins. The envelope has a typical trilamellar structure and is derived from patches of altered cellular membranes. Due to the high lipid content of the envelope the virions are unstable at room temperature and are rapidly inactivated by lipid solvents and detergents (IARC Monographs, 1997). The nucleosome spacing of the EBV DNA-chromatin structure is indistinguishable from that of cellular chromatin (Dyson and Farrell, 1985). Many types of modification are seen in EBV chromatin, which are typical of cellular gene structure. There is extensive methylation of the DNA at CpG dinucleotides (Diala and Hoffman, 1983; Dyson and Farrell, 1985; Kintner and Sugden, 1981; Larocca and
Clough, 1982), the inactive, latent cycle genomes are more completely methylated than productive genomes. In maintenance replication the DNA is on nucleosomes, but in productive replication it is not regularly packed on nucleosomes. The herpesviruses are subdivided based on their host range, site of latent infection, cytopathology, and length of replicative cycle. This subdivision includes alpha, beta and gamma herpesvirinae (Roizman, 1982).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Common name</th>
<th>Sub family</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human viruses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human herpesvirus 1</td>
<td>Herpes simplex virus 1</td>
<td>α</td>
</tr>
<tr>
<td>Human herpesvirus 2</td>
<td>Herpes simplex virus 2</td>
<td>α</td>
</tr>
<tr>
<td>Human herpesvirus 3</td>
<td>Varicella-zoster virus</td>
<td>α</td>
</tr>
<tr>
<td>Human herpesvirus 4</td>
<td>Epstein-Barr virus</td>
<td>γ₁</td>
</tr>
<tr>
<td>Human herpesvirus 5</td>
<td>Cytomegalovirus</td>
<td>β</td>
</tr>
<tr>
<td>Human herpesvirus 6</td>
<td></td>
<td>β</td>
</tr>
<tr>
<td>Human herpesvirus 7</td>
<td></td>
<td>β</td>
</tr>
<tr>
<td>Human herpesvirus 8</td>
<td></td>
<td>γ₂</td>
</tr>
<tr>
<td><strong>Viruses of non-human primates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aotine herpesvirus 1</td>
<td>Herpesvirus aotus type 1</td>
<td>β</td>
</tr>
<tr>
<td>Cercopithecine herpesvirus 5</td>
<td>African green monkey cytomagalovirus</td>
<td>β</td>
</tr>
<tr>
<td>Ateline herpesvirus 1</td>
<td>Spider monkey herpesvirus</td>
<td>α</td>
</tr>
<tr>
<td>Calitrichine herpesvirus 2</td>
<td>Marmoset cytomagalovirus</td>
<td>β</td>
</tr>
<tr>
<td>Cebine herpesvirus 1</td>
<td>Capuchin herpesvirus (AL-5)</td>
<td>β</td>
</tr>
<tr>
<td>Pongine herpesvirus 3</td>
<td>Gorilla herpesvirus</td>
<td>γ₁</td>
</tr>
<tr>
<td>Samirline herpesvirus 1</td>
<td>Marmoset herpesvirus</td>
<td>α</td>
</tr>
<tr>
<td><strong>Bovine viruses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine herpesvirus 5</td>
<td>Bovine encephalitis herpesvirus</td>
<td>α</td>
</tr>
<tr>
<td>Ovine herpesvirus 2</td>
<td>Sheep-associated malignant catarrhal fever</td>
<td>γ</td>
</tr>
<tr>
<td>Caprine herpesvirus 1</td>
<td>Goat herpesvirus</td>
<td>α</td>
</tr>
<tr>
<td>Alcelaphine herpesvirus 1</td>
<td>Wildebeest herpesvirus</td>
<td>γ</td>
</tr>
<tr>
<td>Cevid herpesvirus 1</td>
<td>Red deer herpesvirus</td>
<td>α</td>
</tr>
<tr>
<td><strong>Murid viruses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murid herpesvirus 1</td>
<td>Mouse cytomegalovirus</td>
<td>β</td>
</tr>
<tr>
<td>Murid herpesvirus 4</td>
<td>Mouse herpesvirus strain 68</td>
<td>γ₂</td>
</tr>
<tr>
<td><strong>Gallid viruses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallid herpesvirus 2</td>
<td>Marek’s disease herpesvirus 1</td>
<td>α</td>
</tr>
<tr>
<td><strong>Gruid viruses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gruid herpesvirus 2</td>
<td>Turkey herpesvirus</td>
<td>α</td>
</tr>
<tr>
<td><strong>Ranid viruses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ranid herpesvirus 2</td>
<td>Frog herpesvirus</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: International nomenclature of herpesviruses (Examples of each type, Adapted from IARC Monographs, 1997).
1.2.2 Genome Structure

EBV is a member of the gamma herpesvirinae, its genome is linear double stranded DNA about 172 kilobases in length which circularises inside the host cell. This circularisation is mediated by the joining of terminal repeat (TR) sequences which are about 3-5 in the circularised DNA (Given et al., 1979, Kintner and Sugden, 1979). The genome is subdivided into four regions, these are the short and long unique regions, separated by the major internal repeat (IR), and the terminal repeat (TR). The base composition of the EBV genome is biased to an excess of guanosine (G) and cytosine (C). B95-8 (a strain of EBV) DNA contains about 59.9% GC, and the GC composition is nonuniform throughout the genome, the IR having a higher GC content (Baer et al., 1984). During the seventies the DNA of several EBV strains propagated in marmoset cells was cloned on bacterial plasmids or lambda phages (Colby et al., 1980, Skare and Strominger 1980). The 172 kb sequence of the B95-8 virus was subsequently determined (Baer et al., 1984). EBV was the first herpesvirus whose genome was completely cloned and sequenced.

The complete sequence of the B95-8 genome that is in the European Molecular Biology Laboratory (EMBL) nucleotide sequence library contains an arbitrary number of major IR units (116) and 4 TRs. There may be a distribution of numbers of IR and TR in any culture of EBV-infected cells. No homology exists between the TR and the major IR, which has a large imperfect palindromic sequence of about 100 nucleotides (Cheung and Kieff, 1982). The nomenclature for EBV open reading frames is based on the BamHI-restriction fragment in which they are found. Each fragment is designated a letter, and a number where there is more than one ORF in a particular BamHI restriction fragment. The orientation of the ORF is also defined in the name, e.g. BALF4 - BamH1 fragment A, leftward reading frame ORF 4. See figure 1 of the BamH1-restriction map of the EBV genome.

1.2.3 Variant EBV Strains

The genome structure of a number of EBV strains has been studied and is found to be very similar, the main variation being type variations in the EBNA-2 gene (Dambaugh et
Some laboratory strains display substantial deletions, e.g. B95-8(87), Raji(86), P3HR-1(10), and Daudi(57), these may have arisen in vitro. There are a number of repeats scattered throughout the EBV genome, the TRs are at both ends of the linear form of EBV and mediate the circularisation. The number of repeat units varies between strains, most strains have 3-5, but Raji has up to 12 copies. The major IR unit in B95-8 is 3072 bp, and the number of repeats vary considerably between strains, 4-11. Two major EBV types (1 and 2) have been detected in humans, their genomes are almost identical except for the genes that encode some of the nuclear proteins (EBNA2, EBNA3A, 3B and 3C) in latently infected cells and to the transcription units of the EBER RNAs (Nonoyama and Pagano, 1973, Bornkamm et al., 1980, Arrand et al., 1989). Antibodies to type 2 viruses are more prevalent in the sera of African people than from people in developed countries (Young et al., 1987, Rowe et al., 1989). EBV type 2 infected lymphocytes grow less efficiently in vitro (Rickinson et al., 1987), thus making recovery of this virus type from blood more difficult. In Papua-New Guinea an EBV recombinant that encodes both type 1 and type 2 nuclear antigens was isolated from the blood of a healthy adult (Burrows et al., 1996). In addition to type-specific polymorphism there exist polymorphisms which define different viral strains within type 1 and type 2 (Aitken et al., 1994). These changes can cause amino-acid substitutions in viral proteins and affect peptides that are important for the immune control of viral infection (de Campos-Lima et al., 1993, Lee et al., 1993, de Campos-Lima et al., 1994, Lee et al., 1995, Burrows et al., 1996a).
Figure 1: BamHI-restriction map of the EBV genome and latent transcripts (Adapted from Masucci and Ernberg, (1994) and Rickinson and Kieff (1996))

Abbreviations are explained in the text.
1.3.1 Biology of EBV Infection

Epstein-Barr virus can establish either fully productive (lytic) or nonproductive (latent) infections in target cells. The major target cell types for the virus in vivo are B-lymphocytes and stratified squamous epithelium (See figure 2). There is strong circumstantial evidence to suggest that both lytic and latent infections can be established in cells of either lineage, depending on the precise stage of differentiation of the infected cell (Crawford and Ando, 1986, Greenspan et al., 1985, Li et al., 1992, Rowe et al., 1992). Infection of B cells is mediated through interaction of the viral envelope glycoprotein gp350/220 with the C3d complement component receptor CR2 (CD21) (Fingeroth et al., 1984, 1988). The viral envelope fuses with the host cell membrane resulting in endocytosis, this mechanism involves three other viral glycoproteins gp85, gp25 and gp42 (Li et al., 1995). The glycoprotein gp42 can bind to major histocompatibility complex class II (MHC II), and uses this molecule as a cofactor in the infection of B cells (Li et al., 1997).

Expression of the C3d receptor is not restricted to B cells and identical or related molecules have been detected in T cells, follicular dendritic reticulum cells and possibly some epithelial cells (Reynes et al., 1985, Fingeroth et al., 1988, Timens et al., 1991, Birkenbach et al., 1992, Hedrick et al., 1992, Sinha et al., 1993). EBV has been detected in non-B cell tumours, certain T cell lymphomas and some carcinomas. Table 2 shows the details of some of the EBV-associated malignancies. The mechanism of infection of epithelial cells is as yet not fully understood, some reports suggest that direct infection of nasopharyngeal and cervical epithelial cells with EBV may be possible (Sixbey et al., 1986, Furukawa et al., 1990, Yoshizaki et al., 1994), however, mechanisms to facilitate this process have been devised in most studies to date. Knox et al. (1996) generated stable EBV-infected subclones using CR-2 negative keratinocytes which had been transfected with the gene encoding the B cell EBV receptor. Sixbey and Yao (1992) succeeded in infecting a human colon carcinoma cell line with EBV by coating the virus with gp350-specific polymeric IgA. In certain cell types it seems the absence of CR2 is the main obstacle for EBV infection (Ahearn et al., 1988). It has been shown that EBV induces cell fusion between virus-carrying B cells and other cell types, including T cells and fibroblasts (Bayliss and Wolf, 1980 and 1981), and also that...
endothelial cells can be infected with EBV by co-cultivation with irradiated EBV-carrying B cell lines (Jones et al., 1995). These findings suggest that cell fusion may aid virus entry in the absence of the EBV/C3d receptor.

<table>
<thead>
<tr>
<th>EBV status</th>
<th>NPC</th>
<th>EBL</th>
<th>HD</th>
<th>AIDS-HL</th>
<th>PTLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonality</td>
<td>Mono</td>
<td>Mono</td>
<td>Mono</td>
<td>95%</td>
<td>Mono</td>
</tr>
<tr>
<td>EBV Gene Expression</td>
<td>EBNA1</td>
<td>EBNA1</td>
<td>EBNA1</td>
<td>EBNA1</td>
<td>EBNA1</td>
</tr>
<tr>
<td>LMP1/2</td>
<td>LMP1</td>
<td>LMP1</td>
<td>LMP1</td>
<td>LMP1</td>
<td>LMP1</td>
</tr>
</tbody>
</table>

Table 2: EBV-Associated Malignancies. (NPC - Nasopharyngeal Carcinoma, EBL - Endemic Burkitt's Lymphoma, HD - Hodgkin's Disease, AIDS-HL - Acquired Immune Deficiency Syndrome - Hodgkin's Lymphoma, PTLD - Post-Transplant Lymphoproliferative Disease, Mono - Monoclonal, EBNA - EB Nuclear Antigen, LMP - Latent Membrane Protein, TP - Terminal Protein)

1.3.2 EBV Latent Infection

Circular episomal EBV DNA is detected in the nuclei of blood lymphocytes infected in vitro within 12-16 hr of infection (Hurley and Thorley-Lawson, 1988). It is thought that only DNA repair is required for circularization as significant nucleotide incorporation does not occur until 48 hr post-infection (Sixbey and Pagano, 1985). Most or all of the copies of the EBV latent genome are replicated once in each cell cycle using oriP, early in the S phase by cell DNA polymerase (Adams and Lindahl, 1975, Kaschka-Diench et al., 1976, Shaw, 1985, Adams, 1987). It is most likely that episomal DNA is required for lytic cycle EBV DNA replication, since viral production has not been observed in cells that contain only integrated EBV DNA, and circular DNA copy numbers increase during the lytic infection (Shaw 1985). In the establishment of latent infection, most of the EBV genome undergoes progressive methylation (Kintner and Sugden, 1981, Larocca and Clough, 1982, Perlmann et al., 1982). However, the regulatory domains involved in maintaining latent infection, such as oriP, tend to remain under-methylated (Mnarovitis et al., 1992). Extensive methylation of parts of the genome not expressed in latent infection may help to maintain latency by inhibiting lytic cycle gene expression (Nonkwelo and Long, 1993). Treatment of latently infected cells with drugs that reduce methylation increases the frequency of cells entering the productive cycle (Ben-Sasson and Klein, 1981).
Figure 2: Model of EBV-host interaction during primary infection and persistence. (Adapted from IARC Monographs, 1997)
The maintenance origin of replication, oriP, consists of a series of 30 base pair repeats (7,421-8,042) and a dyad symmetry (9,021-9,133) (Reisman et al., 1985) Both the repeats and the dyad symmetry, which are related in sequence, bind the EBNA1 protein (Rawlins et al., 1985) This binding is required for the origin of replication to function and for transcription enhancer activity, which resides in the 30 base pair repeats (Lupton and Levine, 1985, Reisman and Sugden, 1986) There exists extensive splicing of mRNA in EBV, the genome expresses approximately 90 genes, with two of these giving the EBER RNAs and the rest viral proteins The EBV genome has been compared to that of herpes simplex virus (HSV), cytomegalovirus (CMV), varicella zoster virus (VZV), and herpesvirus saimiri (HVS), however, the latent cycle genes show no obvious relationship to other herpesviruses studied and imply a separate viral or cellular origin By comparing all the EBV reading frames with computer-based libraries a number of homologues were discovered eg HSV ribonucleotide reductase, DNA polymerase, and glycoprotein B (Baer et al., 1984, Gibson et al., 1984, Pellett et al., 1985)

1.3.2.1 Types of Latency

Analysis of EBV latent protein expression in cell culture model systems and in EBV-associated tumours has highlighted the existence of three different forms of latency which are referred to as I, II and III (see table 3) Latency I, seen in BL biopsies is characterised by selective expression of EBNA1 (Epstein-Barr virus nuclear antigen 1) (Gregory et al., 1990, Hitt et al., 1989, Rowe et al., 1987), together with a high copy number of small polyadenylated transcripts, EBER1 and EBER2 (Rymo, 1979, Howe and Shu 1989), and the use of the Fp gene promoter, which allows selective expression of EBNA1 (Sample et al., 1986 and 1991) Latency II, seen in nasopharyngeal carcinoma (NPC) and Hodgkins disease (HD), is characterised by expression of EBNA1, and the latent membrane proteins LMP1 and LMP2A and 2B (Brooks et al., 1992, Busson et al., 1992, Fahraeus et al., 1988, Smith and Griffin, 1991, Young et al., 1988) One or more of the LMP promoters are activated in latency II, resulting in expression of LMP1 and/or LMP2A and/or LMP2B (Kerr et al., 1992) Latency III, seen in infectious mononucleosis (IM) and immunoblastic B-cell lymphomas of the immunosuppressed (PTLD) is characterised by expression of all six EBNA6 (EBNA1,2,3A,3B,3C and LP) as well as LMP1 and 2A and 2B (Kieff and Liebowitz,
There is also expression of cellular genes such as CD23, a ligand for the EBV receptor CD21 (Wang et al., 1987). The pattern of promoter usage in latency III is quite different from that found in latency I and II (Sample et al., 1986; Rodgers et al., 1990). Other viral mRNAs encoded for by the BHRF1 and BARF0 open reading frames (ORFs) have been detected in latently infected cells, but their products are not well characterised (reviewed by Kieff, 1996). EBV-infected proliferating B lymphocytes express activation-associated markers, secrete immunoglobulins and have similar intercellular adherence to lymphocytes that are proliferating in response to antigens, mitogens, or interleukin IL-4 and anti-CD40 (Åman et al., 1986; Hurley and Thorley-Lawson, 1988; Alfieri et al., 1981; Banchereau et al., 1991).

Table 3: Patterns of EBV latent gene expression
(Adapted from IARC Monograph, 1997)

<table>
<thead>
<tr>
<th>Type of latency</th>
<th>Gene product</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>EBERs, EBNA-1</td>
<td>Burkitt’s Lymphoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastric Carcinoma</td>
</tr>
<tr>
<td>II</td>
<td>EBERs, EBNA-1,LMP-1, -2A, -2B, BARF0</td>
<td>Hodgkin’s Disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nasopharyngeal carcinoma</td>
</tr>
<tr>
<td>III</td>
<td>EBERs, EBNA-1,-2, -3A, -3B, -3C, -LP, LMP-1, -2A, -2B</td>
<td>Post-transplant lympho-proliferative disorder</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infectious mononucleosis</td>
</tr>
<tr>
<td>Other</td>
<td>EBERs, EBNA-1, -2</td>
<td>Smooth-muscle tumours</td>
</tr>
</tbody>
</table>

1.3.2.2 EBER1 and 2

The most common viral RNAs in latently infected cells are EBER1 and EBER2, with an estimated $10^7$ copies per cell (Howe and Steitz, 1986). Most EBERs are located in the
nucleus and are associated at the 3' terminus with the cellular La antigen and other cellular proteins (Howe and Shu, 1989) A number of functions have been proposed for the EBERs based on their sequence similarity to adenovirus VAI and VAIL and cell U6 small RNAs (Glickman et al, 1988), however, their function has not yet been elucidated.

1.3.2.3 EBV Nuclear Antigen (EBNA)

Transcription of the latency I EBNA1 mRNAs begins at the Qp promoter (Nonkwelo et al, 1997) Transcription of nuclear proteins is initiated at the Cp and Wp promoters in the BamHI C and BamHI W regions, by polymerase II (reviewed by Rogers et al, 1992), the Fp promoter is a lytic promoter (Nonkwelo et al, 1995 and 1996) In cells that express latency I or II phenotype, the Cp and Wp promoters are extensively methylated and are non-functional (Ernberg et al, 1989, Schaefer et al, 1987) EBNA-LP and EBNA2 are the first viral proteins expressed in infected B lymphocytes, they are detected 12 hr after infection and within 24-32 hr of infection reach the levels maintained in transformed lymphoblastoid cell lines (Alfien et al, 1991) It is thought that EBNA-LP may indirectly or directly up-regulate the expression of autocrine factors required for B cell growth, EBNA-LP and EBNA2 co-operate in the induction of G0 to G1 transition, indicated by induction of cyclin D2, but this mechanism is still unknown (Sinclair et al, 1994)

1.3.2.4 EBNA2

EBNA2 have been found to be essential for transformation and others are important for promoter trans-activating activity (Cohen et al., 1991) (See figure 3). EBNA2 does not interact directly with its responsive elements, the recombination signal sequence binding protein RBP-Jκ, a widely expressed and highly conserved protein acts as a key adapter for transcription regulatory factors of cellular genes (Grossman et al., 1994).

![Figure 3: Schematic representation of EBNA2.](image)

1.3.2.5 **EBNA3A, 3B and 3C**

EBNA3A, 3B, and 3C are encoded by three genes placed tandemly in the EBV genome, the proteins encoded by type 1 and type 2 EBV strains are only 84, 80 and 72% identical in the predicted primary amino acid sequence (Sample et al., 1990). These proteins are located in large nuclear clumps in the nuclear matrix, chromatin and nucleoplasmic fractions but not in the nucleolus (Petti et al., 1990). All three proteins can inhibit EBNA2-activated transcription, probably by their ability to bind RBP-Jκ (Robertson et al., 1995 and 1996; Krauer et al., 1996). EBNA3C up-regulates the expression of CD21 (EBV receptor) mRNA in transfected Burkitt’s lymphoma cells and the expression of LMP1 in Raji cells (Wang et al., 1990; Allday et al., 1993; Allday and Farrell, 1994). EBNA3B up-regulates vimentin and CD40 (involved in cell signal transduction) and down-regulates CD77 (involved in apoptosis) expression (Silins and Sculley, 1994).
1.3.2.6 **EBNA1**

The Epstein-Barr virus nuclear antigen 1 (EBNA1) is a 72kDa multifunctional protein which is a major component of the EBNA complex. It has been shown that EBNA1 is encoded by the BKRF1 open reading frame in the BamK fragment (Hennessy and Kieff, 1983, Dillner *et al.*, 1984). The EBNA1 protein from the B95-8 strain of EBV consists of 641 amino acids and can be divided into three domains. It has a short N-terminal sequence of 89 amino acids that is rich in basic amino acids, followed by a 239 amino acid glycine-alanine copolymer, flanked by basic arginine-rich sequences followed by a highly charged acidic carboxy-terminal sequence (See figure 4). The glycine-alanine copolymer is highly antigenic (Dillner and Kallin, 1988) and similar repeat structures have been found in several proteins including *Plasmodium falciparum* proteins (Berzins *et al.*, 1986) and eukaryotic RNA polymerase II (Corden *et al.*, 1985). During latent infection of human host cells, EBV genomes are maintained as double-stranded DNA episomes that replicate once every cell cycle (Adams, 1987, Yates and Guan, 1991). EBNA1 functions in conjunction with host replication factors and is the only viral protein essential for the replication of the EBV plasmids. EBNA1 dimers interact with the genetically defined latent origin of replication, oriP (origin of plasmid replication) binding to multiple copies of its 18-bp recognition sequence (Rawlins *et al.*, 1985, Reisman *et al.*, 1985). Binding of EBNA1 to the tandem repeats and dyad symmetry sites of oriP enables covalently closed circular DNA molecules to replicate and persist as episomes. The tandem repeat component acts as an EBNA1-dependent enhancer on heterologous or neighbouring promoters in transient transfection assays (Reisman and Sugden, 1986), the dyad symmetry component is stringently required for episome replication. The interaction of EBNA1 with both sites is co-operative and results in higher-order structures that lead to bending of the DNA, distortion of the duplex and looping out of the intervening sequences (Frappier and O’Donnell, 1991 and 1992, Orlowski and Miller, 1991). The C-terminal unique domain carries sequence-specific DNA-binding, dimerisation and transactivation activities (Ambinder, *et al.*, 1991). The amino terminal of the dimerisation domain is important for the formation of macromolecular complexes of EBNA1-homopolymers after association with the DNA templates (Goldsmith *et al.*, 1993), the amino acids 379 - 387 (see figure 4 below) of EBNA1 determines its nuclear location by interacting with a specific protein that is
homogeneously distributed on chromosomes (Ohno et al., 1977; Harris et al., 1985; Petti et al., 1990). Part of EBNA1 is also associated with the nuclear matrix.

**EBNA 1**

| 459-607 | Dimerisation |
| 450-604 | DNA binding |
| 42-76 and 450-641 | Transcriptional activation |
| 379-387 | Nuclear localisation |

Figure 4: Schematic representation of EBNA 1.

Several reports have shown that EBNA1 is not only involved in viral replication but may also play a significant role in the pathogenesis of EBV-related lymphomas (Magrath et al., 1993; Wilson and Levine, 1992). Strong EBNA1 expression has been found in B-cell lymphomas (Oudejans et al., 1996), this observation supports the opinion that in these lymphomas the occurrence of translocations can be facilitated by EBNA1-induced expression of RAG1/2 (Srinivas and Sixbey, 1995), these are V(D)J recombinase activating genes. This may explain why EBV-positive AIDS patients with lymphomas have a worse prognosis than patients with the EBV-negative variant (Kaplan et al., 1995). Differences in the level of expression of EBNA1 in all types of tissue harbouring EBV is most likely due to differential EBNA1 promoter usage (Oudejans et al., 1996).

Regulation of EBNA1 transcription is complex, in EBV transformed lymphoblastoid cell lines and in post-transplant B cell lymphomas EBNA1 and other EBNA mRNAs are derived from highly spliced transcripts that are generated by either the C or W promoters, located on the BamHI C and W fragments respectively (Middleton et al.,
1991) (See figure 1). In other EBV associated malignancies (e.g. BL, Hodgkin’s disease, T cell lymphomas and NPC) there is selective expression of EBNA1 without the expression of the other EBNAs. This occurs as a result of the activation of a different EBNA1 promoter, Q, located on the BamHI Q fragment. In lytic infection EBNA1 transcription is controlled by the BamHI F promoter upstream of the Q promoter (Lear et al., 1992; Schaefer et al., 1995), EBNA1 is the only EBNA that continues to be made during lytic infection.

EBNA1 fails to be processed and presented in conjunction with major histocompatibility complex class I on the surface of tumour cells (Levitskaya et al., 1995), this finding is consistent with the inability of virus-specific cytotoxic T lymphocytes (CTLs) to recognise Burkitt’s Lymphoma tumour cells (Rooney et al., 1985). This means that EBV can persist in normal and malignant cells in vivo without being attacked by the immune system, this ability is thought to be conferred by the glycine-alanine co-polymer domain of the EBNA1 protein. However, a previous study has shown that EBNA1 includes sequences which can be recognised by both polyclonal and clonal CTLs (Khanna et al., 1995). EBNA1 protein from EBV-infected cells may be exogenously endocytosed by professional antigen-processing cells and presented in association with MHC class II molecules, or epitopes generated by serum protease cleavage may be responsible for the activation of CTLs. EBV-infected cells other than B cells could also be involved in the activation of CTLs in vivo.

1.3.2.7 LMP1

LMP1 mRNA is the second most abundant viral transcript in latently infected cells (Fennewald et al., 1984; Sample and Kieff, 1990). LMP1 is an integral membrane protein with a 20-amino acid hydrophilic amino terminus, six hydrophobic alpha helical transmembrane segments and a 200-amino acid carboxy terminus. After phosphorylation, LMP1 is cleaved near the beginning of the carboxy-terminal domain, this results in a soluble 25 kDa product (Moorthy and Thorley-Lawson, 1990 and 1993). Like EBNA1, LMP1 is transcribed during lytic infection and can be induced by treatment with activators of protein kinase C (Rowe et al., 1987a; Laux et al., 1988). LMP1 alters the growth of EBV-negative B lymphocytes and induces many of the changes that are normally associated with EBV infection, these include, cell clumping, increased numbers
of villous projections and vimentin expression, increased surface expression of CD23, CD39, CD40, CD44, MHC class II and the cell adhesion molecules LFA-1, ICAM-1 and LFA-3, and decreased expression of CD10 (Wang et al., 1988; Birkenbach et al., 1989; Wang et al., 1990; Liebowitz et al., 1992; Peng and Lundgren, 1992; Zhang et al., 1994; Kieff 1996). Also LMP1 can up-regulate certain cytokines with B cell promoting activity, such as IL-10 (Nakagomi et al., 1994), and can protect B cells from apoptosis by inducing bcl-2 expression and possibly A20 also (Rowe et al., 1994; Henderson et al., 1991; Martin et al., 1993; Fries et al., 1996). It has also been shown that LMP1 alters the growth of multipotent haematopoietic stem cells and epithelial cells (Dawson et al., 1990; Fähraeus et al., 1990; Hu et al., 1993). EBV recombinants lacking LMP1 are unable to induce growth transformation of primary B cells (Kaye et al., 1993). It seems the mechanism by which LMP1 induces growth transformation involves interaction with cellular proteins that are mediators of cytoplasmic signalling from the family of tumour necrosis factor receptors and thereby induce constitutive activation of the growth, death and necrosis factor-κB signalling pathways of those receptors (Hammarskjold and Simurda, 1992).

1.3.2.8 LMP2A and 2B
LMP2A and 2B are encoded by spliced mRNAs transcribed from the circularised EBV genome across the terminal repeats (Laux et al., 1988), the promoters for LMP1 and 2B form a bi-directional transcription unit containing a common EBNA2 response element, while LMP2A transcription is regulated by a separate EBNA2 response element (Zimber-Strobl et al., 1993). LMP2 co-localises with LMP1 in the plasma membrane of latently EBV-infected B cells (Longnecker and Kieff, 1990; Longnecker et al., 1991).

1.3.2.9 BARFO
In nasopharyngeal carcinoma EBV gene expression includes abundant rightward transcription of the BamHI A fragment, consisting of mRNAs ranging in size from approximately 4.0-8.0 kb. BamHI A transcription is detected at a lower level of expression in EBV-infected lymphoid cells, however, these transcripts have consistently been detected in a wide range of EBV-infected samples, including Burkitt’s lymphoma, parotid carcinoma biopsy samples, type I and type III Burkitt’s lymphoma lines and type III lymphoblastoid cell lines (LCLs). The transcripts found include several distinctly
spliced forms which are 3'-end coterminant and contain the BARF0 open reading frame in the final exon. The 5'-end analysis has confirmed the presence of a previously reported start site and also identified a subset of transcripts of 4.8 kb and larger that initiate further 5' to this site. Also 3'-end analysis identified heterogeneous 3'-end processing in all of the BamHI A mRNAs, resulting in transcripts that either contain the entire BARF0 ORF or are cleaved and polyadenylated 5' to this site (Sadler and Raab-Traub, 1995). The BARF0 ORF has been translated in vitro and is immunoprecipitable with sera from patients with NPC, this is suggestive of its expression in vivo (Gilligan et al., 1991).

In a previous study a peptide corresponding to a region of the putative BARF0 protein was synthesised and used to produce a BARF0-antiserum. This serum was shown to react with 30- and 35-kDa proteins in EBV-positive cell lines and in EBV-positive tumour biopsies (Fries et al., 1997), one of these proteins is known as RK-BARFO. This data indicates that one of the rightward RNA transcripts from the BamHI A region of EBV encodes a protein that is expressed in both latent infection and EBV-infected tumours in vivo. It has been shown that the BamHI A region of the EBV genome is not necessary for transformation of lymphocytes by the virus (Robertson et al., 1994), however it has been detected in two strictly latent LCLs and in neonatal lymphocytes early after infection, suggesting that it functions during latency. It is possible that RK-BARF0 may be involved in latent infection of epithelial cells, presently this cannot be assessed in vitro.

The transcription of the BamHI A region is significantly higher in NPC than in BL or LCLs, this protein has been consistently detected in NPC, which represents latently infected epithelial cells, but not in Oral hairy leukoplakia (OHL), a permissively infected epithelial cell lesion. This supports the suggestion that RK-BARF0 is required in latent epithelial infection but not in viral replication. It does not appear to restrict viral replication as there is an increase in the level of RK-BARF0 in Akata cells following induction of replication. However, its absence in OHL lesions suggests that its function may be restricted to viral replication in B cells. The RK-BARF0 protein was detected in
a group1 BL cell line (Mutu I) and in a BL biopsy, it was thought that EBNA1 was the only viral protein expressed in BL cells. It has been suggested that RK-BARF0 interferes with proteolytic processing and presentation by class I molecules, allowing expression of immunogenic proteins, such as EBNA1, without immune recognition. Identification of the intracellular location of this protein and interacting cellular proteins will help determine the molecular properties of RK-BARF0. It has been shown that the BARF0-encoded protein contains a CTL epitope and that BARF0-specific CTL lines could lyse EBV-negative BL cells stably transfected with the BARF0 gene (Kienzle et al., 1998). As high levels of BARF0 RNA and protein expression have been observed in NPC, this suggests that BARF0-specific CTLs might be of immunotherapeutic value for the treatment of this malignancy. As the levels BARF0 RNA and protein expression are lower in BL cells and LCLs, this may be a contributory factor in the escape of immune recognition from virus-specific CTLs present in the host.

1.3.3 EBV Lytic Infection

Some B lymphocytes spontaneously become permissive for viral replication, in these cells the viral DNA is amplified several hundred-fold by a lytic origin of DNA replication, oriLyt (Hammerschmidt and Sugden, 1988). Cells that have become permissive for viral replication undergo cytoplasmic changes including, margination of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (Gergely et al., 1971), these changes are typical of herpesviruses.

The expression of viral genes associated with productive infection follows a temporal and sequential order. Some viral genes are expressed early after induction, independently of new protein synthesis, these are classified as immediate early genes. Early lytic genes are expressed slightly later, and their expression is not affected by inhibition of viral DNA synthesis, expression of the late genes is blocked by inhibitors of viral DNA synthesis (IARC Monographs, 1997). From in vitro models, it has been shown that all three forms of latency can switch directly into the lytic cycle following activation with phorbol esters, by cross-linking with surface IgM or treatment with calcium inophore (Luka et al., 1979; Takada and Ono, 1989).
Virus replication can be induced by superinfection of cell lines such as Raji, with virus from a latent antigen-defective cell line (P3HR-1) that complements Raji's defect in replicative gene expression (Biggin et al., 1987) Studies with such cell lines has allowed the division of replicative proteins into the early antigen (EA), membrane antigen (MA), and virus capsid antigen (VCA) complexes The EA is divided into restricted (EA-R) and diffuse (EA-D) components (Henle et al., 1971 and 1971a) Serum antibodies to the EA-D component were found to be of prognostic value for patients suffering from infectious mononucleosis or nasopharyngeal carcinoma, while serum antibodies to the EA-R component were of apparent prognostic importance in cases of African Burkitt's lymphoma (Lennette, 1991) The BMRF1 ORF encodes an accessory protein which is a major component of the EA-D complex and is the major early phosphoprotein induced during EBV infection (Epstein, 1984, Pearson et al., 1983) The in vitro activity of the EBV DNA polymerase is dependent on the functional interaction between the catalytic subunit (BALF5 product) and the accessory subunit (BMRF1 product) (Kiehl and Dorsky, 1991, Li et al., 1987, Tsurami et al., 1993 and 1993a) The genes for both components are required for in vivo replication of the viral lytic origin of replication, oriLyt, but not for oriP, which is responsible for the maintenance of latency (Fixman et al., 1992) (Kiehl and Dorsky, 1995)

Most strains of EBV contain two copies of oriLyt, one copy is sufficient for replication (Hammerschmidt and Sugden, 1988) OriLyt overlaps the divergent promoters of the BHRF1 and BHLF1 genes The BMRF1 gene product transactivates the early BHLF1 promoter but does not affect the BHRF1 promoter (Zhang et al., 1996) Combined with the activity of the BZLF1 gene product maximum activation is achieved The BMRF1-response element in oriLyt has been precisely mapped to the essential downstream component (Zhang et al., 1997) The upstream component of the oriLyt is transcriptionally activated by BZLF1 Both the BMRF1 and BZLF1 gene products co-localise within the intranuclear replication compartments during lytic infection (Takagi et al., 1991) and can physically and functionally interact (Zhang et al., 1996) If BMRF1 is bound to the downstream element by interaction with a cellular transcription factor, direct action between BMRF1 and BZLF1 could result in looping of oriLyt Alternatively, BMRF1-induced transcriptional activation of the oriLyt downstream
component could be required for opening the chromatin during the onset of replication (Zhang et al., 1997) However, the exact mechanism by which BMRF1 transcriptionally activates oriLyt is as yet unknown

1.3.3.1 Immediate Early Genes

Three leftward mRNAs are transcribed after P3HR-1 superinfection of Raji or surface Ig cross-linking of Akata cells in the presence of protein synthesis inhibitors The BZLF1, BRLF1 and B'LF4-encoded proteins are potent transactivators of early EBV gene expression (Takada and Ono, 1989, Marschall et al., 1991, Kieff, 1996) Two early promoter regulatory elements with left and right duplications that include the origins of lytic viral DNA replication are co-ordinately up-regulated by BZLF1 and BRLF1 (Hudewentz et al., 1982, Hummel and Kieff, 1982, Freese et al., 1983, Nuebling and Mueller-Lantzsch, 1991) The R protein is a DNA sequence-specific acidic transactivator that has distant homology to c-myb (Gruffat and Sergeant, 1994, Kieff, 1996) The Z (ZEBRA) protein is spliced and consists of three exons which have different functions The first 167 amino acids make up the trans-activating domain (Taylor et al., 1991, Chu and Carey, 1993), the second exon confers the ability to interact with AP1-related sites in DNA and also targets Z to the nucleus (Mikaëlian et al., 1993) The third exon is required for interaction of Z with p53 (Zhang et al., 1994a)

1.3.3.2 Early Genes

The early genes are expressed when the lytic cycle is induced in the presence of DNA synthesis inhibitors, several of these genes are linked to DNA replication These include DNA polymerase (BALF5), the major DNA-binding protein (BALF2), ribonucleotide reductase (BORF2 and BARF1), thymidine kinase (BXLF1) and alkaline exonuclease (BGLF5), which are distributed through the unique long domain of EBV DNA (Kieff, 1996) The BALF2 and BHRF1 gene products are two very abundant early proteins BHRF1 mRNA has been identified in strictly latently infected cells, however, the protein it encodes can only be found in cells in early lytic infection (Austin et al., 1988, Alfieri et al., 1991) The BHRF1 protein is expressed in moderate abundance, has extensive collinear homology with bcl-2 (Pearson et al., 1983, Austin et al., 1988), and can protect
EBV-negative Burkitt’s lymphoma cells from apoptosis (McCarthy et al., 1996). However, EBV recombinants lacking the BHRF1 ORF are capable of initiating and maintaining cell growth transformation and can also enter the lytic cycle and produce virus (Lee and Yates, 1992; Marchini et al., 1991).

1.3.3.3 Late Genes

The late genes code for structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress. Of the non-glycoproteins, the major nucleocapsid protein is probably encoded by BcLF1, BNRF1 encodes the major external non-glycoprotein of the virion, and BXRF1 is likely to encode a basic core protein (Kieff, 1996). The BFRF3 ORF encodes a highly basic viral structural capsid protein, p18 VCA, which is strongly immunogenic in humans (van Grunsven et al., 1994), this will be discussed later in further detail. It does not appear to have any sequence homologues among other herpesviruses. The known EBV glycoprotein genes are BLLF1 (gp350/220), BALF4 (gp110), BXLF2 (gp85), BILF2 (gp55/80), BDLF3 (gp100-150) and BZLF2 (gp42) (Kieff, 1996). The membrane antigen complex is involved in mediating virus binding to the B lymphocyte receptor CR2 (Nemerow et al., 1985; Tanner et al., 1987) and consists of at least four major EBV-induced glycoproteins, gp350/220, gp250/200, gp85, and gp78/55 (Mackett et al., 1990). Gp85 is a relatively minor viral protein which is important in fusion between the virus and cell membranes (Miller and Hutt-Fletcher, 1988), gp85 and gp350/220 are processed through the Golgi apparatus and are found on the virus and in the plasma membrane of lytically infected cells (Gong and Kieff, 1990; Kieff, 1996). The membrane antigen (MA) glycoprotein is encoded by nucleotides 1784-4504 of the BamHI L fragment of the EBV genome (Zhang et al., 1991). gp350 is a 907 amino acid protein with 37 putative N-glycosylation sites. Both gp350 and gp220 are derived from a single gene by splicing, without a change in reading frame (Beisel et al., 1985). The carbohydrate moiety of gp350 was found to be made up of both O-linked and N-linked types and to constitute about 50% of the molecular mass. The high carbohydrate content of gp350 appears to confer resistance to proteolysis (Morgan et al., 1984). The MA is a component of the virus envelope (Pearson et al., 1971), and neutralising determinants are included in gp350/220, gp250/200, and gp85 (Hoffman et al., 1980; Qualtiere et al., 1982; Strnad et al., 1982).
The viral capsid antigen is abundantly expressed in cell lines undergoing productive infection, and has polypeptide and glycoprotein components ranging in size from 18 to 200 kDa, with a 143-kDa polypeptide (BNRF1 reading frame) (Thorley-Lawson 1982) being the major component. It may be composed of at least seven proteins (Dillner and Kallin 1988), of which the capsid protein encoded by ORF BcLF1 (Vroman et al., 1985) and a 36 kDa protein encoded by BGLF2 (Chen et al., 1991, Seibl and Wolf, 1985) have been described in detail. Another VCA protein, gp110, is encoded within the BALF4 ORF (Takada et al., 1982) and is one of the most abundant late EBV glycoproteins, which is localised to the inner and outer nuclear membrane and to the cytoplasmic membranes frequently surrounding enveloped virus (Emmi et al., 1987, Gong et al., 1987, Gong and Kieff, 1990).

Two immunologically dominant VCA proteins with molecular sizes of 18 and 40 kDa have recently been identified (van Grunsven et al., 1993a). Both of these proteins are structurally associated with the viral capsid and are recognised by antibodies from almost all EBV carriers. Sera from most IM patients already contain detectable levels of IgG to p18 and p40 at early stages of disease, which increase rapidly in titre and remain constant following recovery (van Grunsven et al., 1993). The serological profile against EBV-VCA proteins has proved to be useful in the differential diagnosis of EBV-related diseases (Ooka et al., 1991), such as IgA for diagnosis of NPC and elevated IgG titres in BL (Desgranges and De The, 1987), oral hairy leukoplakia (Greenspan et al., 1985), and Hodgkin disease (Mueller et al., 1989). The VCA antigens are gene products whose synthesis is blocked by inhibitors of the viral DNA replication. Only part of these VCA-class antigens may form structural components of the EBV virion, the major VCA antigen components p18 and p40 belong to this class of structural VCA antigens (van Grunsven et al., 1993a).

The late BCRF1 gene is located in the middle of the EBNA regulatory domain between oriP and the Cp, and the protein it encodes has almost 90% collinear identity in amino-acid sequence with human IL-10 (Moore et al., 1990, Vieira et al., 1991, Toutou et al., 1996). BCRF1 also has most of the activities of human IL-10 including negative regulation of macrophage and NK cell functions and inhibition of IFN-γ production.
Therefore, virally expressed IL-10 may have a local effect on these responses to reactivated infection.

1.4 EBV and Disease

1.4.1 Self-limiting Disease - Infectious Mononucleosis

The classical clinical syndrome associated with primary EBV infection is infectious mononucleosis (IM), commonly known as glandular fever, which is a benign self-limiting disease. In most cases the disease runs its course within a few weeks. Primary infection during early childhood appears to result in mild or no clinically detected symptoms (Henle and Henle, 1970). In contrast, infection during or after adolescence can give rise to IM in up to half of the infected individuals (Henle and Henle, 1979). Hormonal changes and maturation of the immune response are thought to be possible reasons for this maturation-related incidence of disease. There is a 30-50 day incubation period, followed by a 3-5 day period where mild symptoms are experienced, these include headache, malaise and fatigue. In more than 80% of cases a sore throat will occur during the first week. Fever with temperatures reaching 39.5°C or higher lasts for about 10 days and then falls gradually over an additional 7-10 days. Patients gradually develop generalised lymphadenopathy. Splenomegaly is observed in about 50% of patients in the second and third weeks. Hepatosplenomegaly, rash and several central nervous system syndromes may also occur. Fatal mononucleosis usually occurs in individuals with an underlying genetic defect, such as X-linked lymphoproliferative syndrome (Miller, 1990). Patients with this disorder suffer from an immune defect that makes them sensitive to EBV-induced diseases, most infected men die by the age of 40 (Purtilo, 1991). Patients with XPL may develop Burkitt’s lymphoma carrying \textit{c-myc} translocations (Purtilo, 1991).

Infectious mononucleosis represents a benign, lymphoproliferative disorder with prominent expansion of the paracortex of lymphoid tissues by numerous activated B blasts. Morphological, molecular and tissue culture studies have shown the proliferation of EBV-infected polyclonal blasts, accompanied by the growth of activated T cells (Svedmyr and Jondal, 1975; Tosato \textit{et al.}, 1979; Syedmyr \textit{et al.}, 1984; Brown \textit{et al.}, 1991).

The acute phase of virus infection is characterised by a well-defined serological pattern. There is the absence of antibodies to EBNA and the presence of IgM antibodies to structural components of the virion, anti-VCA (viral capsid antigen) and anti-MA (antimembrane or envelope antigen). Antibodies to early components of the viral replication cycle, early antigens (EA) are also readily detected (Henle and Henle, 1979). IgM antibodies to VCA evolve quickly with infection, persist for weeks to months, and do not reappear. Their detection is presumptive evidence of recent primary infection. (See section 1.5 below). Antibodies to EA of the diffuse or restricted types develop in most primary infections and wane with time. (Horowitz *et al.*, 1985).

Infectious mononucleosis follows a type III EBV latency pattern of gene expression, however a small number of cells also express lytic cycle antigens, such as BZLF1 and EA. Plasma cells may sustain full replication of EBV and may represent a cellular source of infectious virus in the saliva of patients with IM (IARC Monographs, 1997).

Infectious mononucleosis is a self-limited disease, with few exceptions, however, a number of complications may develop depending on the immunopathologic responses to the virus. Severe autoimmune haemolysis, airway obstruction from grossly enlarged tonsils, splenic rupture, and encephalitis have been observed in otherwise healthy patients. Agranulocytosis, aplastic anaemia, and other rare features of acute progressive EBV infection can arise in healthy patients. These symptoms indicate a deficiency in cellular immunity in the patient (Straus, 1992). Several central nervous system syndromes have been described in association with IM. These include aseptic meningitis, encephalitis, acute psychosis, coma transverse myelitis, acute cerebellar syndrome, and infectious polyneuritis (Grose *et al.*, 1975). This may be partly due to
invasion and proliferation of EBV-infecting B cells into the central nervous system, lymphoid cells harbouring EBV and EBV-specific antibodies have been found in cerebrospinal fluid (Schiff et al., 1982).

Chronic infectious mononucleosis is a rare, heterogeneous, and poorly understood syndrome that arises in previously well men and women (Schooley et al., 1986; Straus, 1988). Reactive adenopathy and hepatosplenomegaly, uveitis, pneumonitis and polyneuropathy are characteristic of this syndrome. Progressive cellular and humoral immune deficiencies evolve in the course of follow-up, it is therefore impossible to determine if active infection causes the impairment of the immune system or if the infection persists because of immune deficiency (Straus, 1992a). A number of patients with this syndrome have been studied, their illnesses lasted two to fourteen years and three died during the study period. There exists a second form of chronic IM which is less severe, it is often referred to as chronic fatigue syndrome, and is far more common. Some patients have elevations of antibody titres to EAs and a few lack antibody to EBNA1, however the link with EBV infection has not been confirmed (Straus, 1988). The symptoms include fatigue, chronic pharyngitis, tender lymph nodes, headaches, myalgia and arthralgias, the symptoms are recurrent and prolonged (Miller, 1990). Chronic fatigue syndrome (CFS) may arise in individuals in the absence of an EBV infection, it may be due to another herpesvirus or as in many cases there is no virus detected. This indicates that EBV is not the causative agent of CFS, but may contribute to it’s development in some cases.

1.4.2 Malignant Disease

1.4.2.1 Burkitt’s Lymphoma

Burkitt’s lymphoma (BL) is the most common childhood cancer in certain parts of equatorial Africa and Papua New Guinea, with an annual incidence of more than 50 cases per million children, below the age of sixteen. Burkitt’s lymphoma now accounts for 30-70% of childhood cancers in equatorial Africa. The high incidence of BL in these locations is associated with geographic and climatic features coincident with haloendemic malaria (Burkitt, 1983 and 1969; O’Connor 1970). Burkitt’s lymphoma is a poorly
differentiated malignant lymphoma in which the tumour cells show little variation in size or shape. The tumour cells are monoclonal B lymphocytes and they contain characteristic chromosomal translocations (Manolov and Manalova, 1972; Manalova et al., 1979) (Rowe and Gregory, 1989). In the areas of Africa where BL is endemic, more than 90% of the tumours contain EBV DNA and express EBNA (Geser et al., 1983), the number of genome copies per cell has been found to range from 10 to 113 (Reedman et al., 1974; Lindahl et al., 1974). However, in parts of the world where BL is sporadic (Western Europe and the Americas), only about 15-20% of BL tumours contain EBV DNA. In temperate regions in South America, such as Argentina and Chile, the rate of EBV association is lower than in tropical regions in the north of the continent, however, this may be accounted for by the differences in climate and socioeconomic circumstances. This difference in the presence of EBV DNA in tumours indicates that EBV is not essential for formation of the tumour, therefore, EBV may have no direct role in the pathogenesis of BL, but may simply increase the risk of development of BL by virtue of it’s ability to immortalise B cells (including the cell population that gives rise to Burkitt’s lymphoma) (Klein, 1979). This hypothesis is consistent with the lack of expression of EBV latent genes (e.g. EBNA2, EBNA3 and LMP) known to be necessary for the transformation of B cells (Alfieri et al., 1991; Woisetschlaeger et al., 1991). The only latent gene invariably expressed in Burkitt’s lymphoma, EBNA1, has never been shown to have transforming functions (Rowe et al., 1987, 1988 and 1992; Sample et al., 1991; Schaefer et al., 1991). The sporadic BL presents at a slightly later age and frequently involves abdominal and lymphoid tissue but rarely involves the jaw, the high incidence BL frequently involves the jaw and the abdominal viscera but rarely lymphoid tissues. (Magrath et al., 1993). In series of cases of BL in Uganda, 70% of children under 5 years of age and 25% of patients over 14 had jaw involvement (Burkitt, 1970a).

Burkitt’s lymphoma is invariably of B cell origin, the presence of surface immunoglobulin (Ig), was first shown in 1967 (Klein et al., 1967). The surface Ig is usually IgM, but IgG and IgA are occasionally present. The surface antigens expressed are CD10, CD19, CD20, CD22, CD77 and CD79a (Harris et al., 1994). Burkitt’s Lymphoma cells also express low levels of HLA class I adhesion and activation molecules such as CD54, CD11a/18 and CD58 (Masucci et al., 1987; Billaud et al., 1989; Andersson et al., 1991).
There are three known co-factors involved in the pathogenesis of high incidence BL, these are malaria, chromosome translocation and EBV. There are a number of factors which suggest that malaria is a co-factor in the development of Burkitt's lymphoma, these include, (a) The incidence of BL correlates within countries and internationally with the incidence of malaria and with parasitaemia rates, (b) The age at which peak levels of anti-malarial antibodies are acquired (5-8 yr) corresponds to the peak age incidence of BL, (c) Individuals in urban areas where malarial transmission rates are lower also have lower incidence of BL and (d) There is some evidence for a seasonal variation in the onset of BL and for time-space clustering, (Morrow, 1985) It is thought that malaria plays a part via immunosuppression, resulting in uncontrolled B-cell proliferation, which is partly due to EBV. Chronic malaria leads to a shift in the helper T cell response towards Th2 cells (von der Weid and Langhorne, 1993) Th2 cytokines, such as IL-10, suppress the humoral arm of the immune response, support the early steps of B cell immortalisation by EBV (Burdin et al., 1993), and suppress CTL function. Consequently, the number of B lymphocytes latently infected with EBV increases, while the ability of T cells to suppress the outgrowth of EBV-infected lymphoblastoid cells is impaired (Gunapala et al., 1990, Moss et al., 1983, Whittle et al., 1984, Lam et al., 1991) Young children at greatest risk suffer multiple bouts of clinical malaria every year and during acute attacks their T-cell immunity to EBV infected B cells is significantly impaired (Moss et al., 1983, Whittle et al., 1984) It has been proposed that the increased frequency of B cell mitosis, arising from the combined effects of malaria and EBV infection, increases the chances of random chromosomal aberrations, including the specific translocations of BL (Klein, 1983 and 1987) The c-myc protooncogene is activated by the chromosomal translocation, this activation is a co-factor in the development of BL, shown in studies with transgenic mice. Deregulated c-myc alone is thought to be insufficient to induce the fully malignant phenotype but it causes a prelymphomatous state where abnormal polyclonal proliferation and accumulation of B cells at a particular stage of differentiation occurs (Langdon et al., 1986) The translocation is likely to occur at one of the points at which the Ig gene complex is genetically unstable, possibly at the pre-B stage during VDJ joining. Geographical studies of the myc/lg chromosomal breakpoint locations have shown a different pattern in every region studied (Magrath et al., 1993) This suggests that BL consists of several
subtypes which occur in different proportions in different regions of the world, and that these subtypes are environmentally determined.

Due to the down-regulation of EBV latent proteins BL cells have an advantage in escaping virus-specific immunosurveillance, yet can contribute to the oncogenic process. All the latent genes may be expressed during the initial infection of the BL progenitor cell but are subsequently down-regulated as the growth transforming property of the virus is replaced by the activity of deregulated c-myc. There is constant expression of EBNA1 and of the EBERs, this implicates an essential role for these gene products in the maintenance of the malignant state of BL cells (Rowe and Gregory, 1989). It is thought that EBV may be essential in pathogenesis in some molecular varieties of BL but not others.

Previous work has shown that endemic tumours, associated with EBV, have approximately 75% of tumours in which the breakpoint on chromosome 8 is distant from c-myc and this led to the investigation of the possibility that EBNA1 may collaborate with the myc/Ig translocation in the pathogenesis of BL. Specific antisense inhibition of EBNA1 in Raji cells has been shown to cause failure in proliferation and cell death, the growth inhibition was accompanied by a decrease in both c-myc and \( \mu \) heavy chain expression. Transfection of an EBV cell line with EBNA1 caused increased transcription when also transiently transfected with an immunoglobulin enhancer and a part of c-myc. Electrophoresis mobility shift assays have shown that EBNA1 acts directly with a cellular protein rather than inducing expression of another gene (Magrath et al., 1993). This evidence suggests that EBNA1 can participate in the deregulation of c-myc, when c-myc is juxtaposed to an immunoglobulin enhancer. EBV infection of a large number of precursor B cells greatly increases the chance that a translocation and EBV infection will happen in the same cell, explaining why EBV infection at an early age is associated with a higher proportion of EBV-associated BL, and accounts for the risk of children with anti-VCA titres above the mean for their age group in Africa (Geser et al., 1983). The combination of EBV and malaria further increases the risk. HIV is another environmental factor which can influence the occurrence of BL, like malaria, there is evidence that HIV impairs cell-mediated immunity against EBV infected B cells (Birx et al., 1986). HIV
may contribute by inducing severe immune suppression, leading to a loss of EBV-specific T cell immunity, or by chronic stimulation of the B cell system (IARC Monographs, 1997).

1.4.2.2 Nasopharyngeal Carcinoma

The etiologic link between nasopharyngeal carcinoma (NPC) and EBV was first suggested based on serological evidence (Old et al., 1966). This association was subsequently confirmed by EBV nucleic acid hybridisation studies on NPC biopsy material and by demonstrating that EBV DNA was present in a majority of NPC tumours (zur Hausen et al., 1970; Nonoyama et al., 1973; Wolf et al., 1973; Klein et al., 1974). It has been shown by Southern blot hybridisation that EBV DNA in NPC biopsies is clonal, arising from a single EBV-infected cell (Raab-Traub and Flynn, 1986). EBV is present in the cell at the time of carcinogenic transformation, this suggests that the virus contributes to the transformation event. NPC is a rare malignancy in most parts of the world, however, it is highly prevalent among southern Chinese (Ho, 1972), this indicates that genetic or environmental factors may contribute to the development of NPC. Southern Chinese are an ethnically distinct population and they may be related to Aluet Indians, another high-risk group who are ethnically distinct. The Inuits and other natives of the Arctic region have high rates of NPC also (Albeck et al., 1992; Nutting et al., 1993), as do many indigenous peoples of Southeast Asia, including Thais, Vietnamese, Malays and Filipinos (Parkin et al., 1997).

In about half the cases of NPC the presenting sign is a cervical mass resulting from spread to regional lymph nodes. Other symptoms may include nasal obstruction, postnasal discharge or epistaxis and possibly impairment of hearing, tinnitus or otitis media. NPC may metastasise to the skeleton, the spine, the liver, lung and skin as well as to the peripheral lymph nodes (Miller, 1990), as a result of metastasis NPC is often identified only after dissemination.

EBV infection is an essential step in the progression to malignancy. The nasopharyngeal epithelium becomes infected early in life by EBV. BARF0, LMP-2, EBER and EBNA1
are always expressed in NPC cells, while LMP1 is expressed in a variable proportion of
tumour cells (Fähraeus et al., 1988, Young et al., 1988, Brooks et al., 1992, Gilligan et
al., 1991, Sbih-Lammali et al., 1996) LMP1 has strong growth stimulating effects in
vitro and may have similar effects in the nasopharyngeal epithelium. As the cells are
stimulated to divide, EBNA1 ensures that the viral genome will replicate and be
distributed to progeny cells. The early genes BALF5, BZLF1, BMRF1, EA-D and BHRF
(EA-R) are occasionally detected in a few cells, early proteins such as ribonucleotide
reductase, BZLF1, BMRF1 (EA-D) and BHRF1 (EA-R) can be detected with
monoclonal antibodies (Luka et al., 1988, Lung et al., 1989, Cochet et al., 1993) To
reach full malignant potential the dividing nasopharyngeal cells may acquire cellular
genetic alterations, the risk for which may be increased by exposure to environmental
carcinogens such as volatile nitrosamines in salted fish (Liebowitz, 1994)

Ingestion of Cantonese style salted fish, correlates with increased risk for NPC (Ho,
nitrosamines have been detected in Chinese salted fish, however, their precise role in
inducing NPC has yet to be determined (Huang et al., 1981) Studies suggest that age at
exposure is an important co-determinant of risk, earlier age at exposure being associated
with higher risk for disease (Armstrong et al., 1983, Ning et al., 1990, Yu et al., 1988,
Zheng et al., 1994) Fumes, smoke, dust, formaldehyde, tobacco, alcohol, herbal drugs,
anti-mosquito coils and Chinese nasal oil are other environmental factors whose influence
has been assessed in relation to the risk of NPC development. In general it appears that
each of the above factors may significantly increase the risk for NPC (Armstrong et al.,
1983, Blair et al., 1986, Zhu et al., 1995, Vaughan et al., 1996, Zheng et al., 1994a,
West et al., 1993, Yu et al., 1990) Early evidence for a genetic determinant among
Chinese was an HLA-associated risk for NPC (Simons et al., 1974 and 1976) The
presence of both A2 and BW46 antigens was associated with a twofold increased risk for
NPC among Chinese in Singapore, Malaysia, Hong Kong and Guangzhou (Simons et al.,
1978, Chan, 1983a) Other HLAs have also been shown to be associated with NPC in
selected populations Familial aggregation has been reported in diverse populations,
ranging from high risk populations such as the southern Chinese to low risk Caucasians
This phenomenon can be a result of shared genes, shared environments or both (IARC Monographs, 1997)

Early studies have shown that patients frequently possess elevated serum antibodies to two EBV lytic cycle antigen complexes, viral capsid antigen (VCA) and early antigen (EA) (Henle and Henle 1976, Ho et al., 1976) Serum detection of these antibodies is a routine diagnostic test for NPC in South-east Asia (Yip et al., 1996) It has been shown that the ZEBRA protein can be expressed occasionally in a subpopulation of NPC cells (Cochet et al., 1993), and NPC patients also have serum antibody to ZEBRA (Joab et al., 1991) It was found that detection of antibodies to early lytic antigens (ZEBRA and EA) could be of greater prognostic value than those to the late ones (VCA and MA) or latent antigens (LMP2, EBNA 1 and 2), and that IgG antibodies may be more prognostic than IgA antibodies (Yip et al., 1996)

1.4.2.3 Hodgkin’s Disease

EBV association with Hodgkin’s disease (HD) was first suggested by serological studies, and subsequently analysis of tumour tissue extracts (Weiss et al., 1987) Studies of over 1000 patients over a 2 year period showed on average 50% positivity for EBV, by various techniques, in the Reed-Sternberg (RS) and Hodgkin cells (Joske and Knecht, 1993) PCR has been used to detect EBV DNA in HD tissues, showing a high percentage of positive cases, however, it has been necessary to use in situ techniques to assess the significance of PCR positive results (Pallesen et al., 1993) Viral replication is only seen occasionally in RS cells in HD cases, this shows that in most EBV-positive HD cases viral infection is strictly latent (Pallesen et al., 1991) Type II EBV latency is detected in RS cells, they are EBER-positive, EBNA1-positive and LMP1-positive (Joske and Knecht, 1993) All cases showing EBER1 expression also demonstrate LMP1 expression, this occurs only in a proportion of RS cells At the transcriptional level LMP1 expression is associated with a high viral burden, this suggests that expression of this oncogene is of biological significance (Joske et al., 1992), however, LMP1 positivity does not affect prognosis (Vestlev et al., 1992, Fellbaum et al., 1992) It is thought that the RS cells have acquired the ability to tolerate high levels of LMP1, the levels
measured are toxic to normal cells (Hammerschmidt et al., 1989, Knecht et al., 1993). Previous work has shown there may be geographical variation in EBV positivity rates worldwide, similar to the situation with Burkitt's lymphoma (Ambinder et al., 1993, Chang et al., 1993). Up to 50% of cases in western countries and up to 100% in some other populations carry the virus (Armstrong et al., 1992, Weinreb et al., 1996). Patients with IM have a fourfold increase in risk for developing Hodgkin's disease (Muñoz et al., 1978). Cases of HD arising in immunosuppressed individuals are almost invariably EBV-associated, and regression of EBV-positive cases of HD has been reported after restoration of the immune system (Berger and Delecluse, 1993). This provides further evidence for an etiological role for EBV in the pathogenesis of Hodgkin's disease.

Hodgkin's disease has previously been classified as four major types, nodular lymphocyte predominant, nodular sclerosis, mixed cellularity and lymphocyte depleted. However, there is increasing evidence to suggest that HD is a heterogeneous group of diseases, this prompted inclusion of HD in the Revised European-American Lymphoma (REAL) classification (Harris et al., 1994). Hodgkin's disease usually arises as a unifocal lesion in cervical lymph nodes followed by spread of the tumour to adjacent lymph nodes, giving rise to enlarged nodes. The tumour spreads through the lymphatic channels and other organs can become involved, the preferential sites of involvement include the spleen and distant nodes. As the disease becomes more aggressive other organs are involved, including the kidneys and liver (Kaplan, 1980). Much evidence suggests that the risk for HD in young adulthood through middle age is associated with higher education, higher social class, fewer siblings, less crowded housing and early birth rank. These factors lead to susceptibility to late infections with the common childhood infections which tend to be more severe than those in younger children (IARC Monographs, 1997). Because HD in childhood occurs primarily in developing countries, the children at risk would appear to be of lower social class and thus are infected earlier by EBV.

The role of EBV in Familial Hodgkin's disease (FHD) has also been studied by Lin, et al., 1996. As with sporadic HD the causes are unclear, HLA phenotype and disease susceptibility has been explored, however, this may account for only a fraction of cases. Environmental factors may also be involved, these require further investigation. FHD
patients were found to have higher geometric mean antibody titres (GMTs) to the viral capsid antigen (VCA) and early antigen (EA-D), and 28% had EBER1 expressed in the RS cells. However, lack of concordance of EBER1 expression and EBV serology in FHD cases in the same family suggest that EBV does not play an important role in FHD. EBV could be important in a subset of FHD with a specific genetic predisposition that is not yet identified (Lin et al., 1996).

1.4.2.4 AIDS-Related EBV Infection

Development of lymphomas is a common occurrence in patients carrying Human Immunodeficiency virus (HIV), being 60 times more common than the general population (Beral et al., 1991). AIDS-related lymphomas (ARLs) may show EBV infection as do other immunodeficiency-associated lymphomas, suggesting a direct oncogenic effect of EBV infection in B cells, when physiological immune regulation and natural defence barriers have broken down. Four kinds of EBV-positive lymphomas are seen in AIDS patients (Anonymous, 1991), these are B cell immunoblastic lymphoma, closely resembling PTLs, a small cell, or Burkitt-like lymphoma, primary central nervous system (CNS) lymphoma, and Hodgkin’s disease. EBV gene expression in these lymphomas is the same as non-HIV-associated lesions. Burkitt’s lymphoma is EBV-positive in 20% of cases (Beral et al., 1991), and the immunoblastic lymphoma is EBV-positive in 50% of cases (Hamilton-Dutoit et al., 1991). Studies using in situ hybridisation have shown that 100% of CNS lesions are EBV-positive, all of these being of B cell phenotype (DeAngelis et al., 1992, Morgello 1992, MacMahon et al., 1991). A number of case studies have shown that 88% of HIV-associated HD patients are EBV-positive (Uccini et al., 1990, Moran et al., 1992, Audouin et al., 1992). There tends to be less lymphocytic infiltration in HIV-positive cases as compared to HIV-negative cases, this is thought to be due to the T-cell depletion and breakdown of cellular immunity (Pelstring et al., 1991). It has been shown that HIV-positive individuals with HD are more likely to have advanced and extra-nodal disease, not to respond to therapy, and to die of opportunistic infection than those with HD alone (Ames et al., 1991, Knowles et al., 1988).
Most AIDS-related non-Hodgkin’s lymphomas appear to be monoclonal with respect to their antigen receptor genes and to the EBV episomes (Ballermann et al., 1993, Delecluse et al., 1993, Shibata et al., 1993) However, rare cases may be polyclonal and thus resemble post-transplant lymphoproliferative disorder (Delecluse et al., 1993) AIDS-related B-cell lymphomas consistently lack the HIV genome, therefore, a direct contribution of HIV to tumorigenesis beyond suppression of the immune system is unlikely (Knowles, 1993) Disturbances in EBV-specific immunity are a pathogenic factor in the development of some EBV-associated, AIDS-related non-Hodgkin’s lymphomas As the central nervous system is less accessible for immunosurveillance it is particularly susceptible to the development of AIDS-associated lymphomas (MacMahon et al., 1991)

The clinical, molecular and viral features of AIDS-related BL suggest a pathogenesis similar to that of sporadic BL The primary event appears to be chronic stimulation of the immune system, leading to polyclonal B cell hyperplasia, which may be initially driven by HIV infection The low rate of EBV infection in these lymphomas suggests that the function of the virus could be substituted by other factors, which as yet remain unidentified (IARC Monographs, 1997)

Many AIDS patients develop oral hairy leukoplakia (OHL) tongue lesions, which contain a high level of EBV replication (Greenspan et al., 1985) The lesion usually presents as a white patch that often appears corrugated or even hairy, most commonly on the lateral margin of the tongue Approximately 20% of otherwise asymptomatic HIV patients develop OHL, this increases as the immune status, CD4 cell count and clinical condition deteriorate (Greenspan et al., 1995) A significant proportion of patients with OHL and without AIDS subsequently progress to AIDS fairly rapidly (Katz et al., 1992) Oral hairy leukoplakia can be induced to regress by treatment with acyclovir, indicating that this lesion is caused by EBV infection (Resnick et al., 1988)

A combination of Northern blotting and sequencing of clones from a cDNA library, constructed using RNA isolated from OHL, has been used to study EBV gene expression
in this AIDS-associated lesion (Lau et al., 1993) No expression of EBNA1, EBNA2 or EBNA3A RNA was detected on the OHL cDNA library BdRF1, BCRF1, gp350, and the BARF0 ORF were selected for detailed study as these are genes expressed in the EBV productive cycle, there is no detectable latent phase in OHL Analysis of BZLF1 RNAs from this library have also been studied (Lau et al., 1992) Five full length cDNAs and one partial cDNA covering BdRF1, five cDNAs covering BCRF1, four partial cDNAs covering the 3’end of the gp350 gene, one cDNA spanning part of the BKRF1 ORF, and several cDNAs covering the BARF0 ORF were isolated (Lau et al., 1993) Previously RNAs crossing BARF0 have only been reported from latent EBV infections in NPC and lymphocytes (Gilligan et al., 1990, Hitt et al., 1989, Karran et al., 1992) There were unexpected differences in the relative abundance of cDNA clones isolated, this is thought to reflect the abundance of their respective mRNAs

1.4.2.5 Post-Transplant Lymphoproliferative Disease and EBV

In the early 1980’s EBV was first associated with post-transplant lymphomas (Crawford et al., 1980) Lymphoproliferative disease (LPD) is a significant cause of morbidity and mortality in these patients as lifelong immunosuppression is essential for graft survival Incidences of LPD vary with different types of organ grafts, heart-lung recipients have the highest reported incidence at 33% (Randhawa et al., 1989), while bone marrow transplant recipients have a low rate of incidence The general consensus is that LPD development in recipients of solid-organ transplants are of host-origin, while those occurring in bone-marrow transplant patients are of donor origin (Zutter et al., 1988, Chadburn et al., 1995, Weissman et al., 1995) Lack of previous exposure to EBV has been shown to carry an increased risk of LPD development (Ho et al., 1988) Serological data from LPD patients shows that approximately 50% of cases are EBV seronegative at the time of transplant and have a recent primary infection just prior to lymphoma development (Ho et al., 1985, Thomas et al., 1990) The majority of LPD tumours show the full latent viral gene expression (EBNA1-6, LMP, TP1 and 2), this implicates EBV directly in their aetiology

36
The clinical features of LPD can be divided into two types, IM-like illness and lymphoma-like presentation. The IM-like illness is usually presented by young patients within a year of transplant, the symptoms are generalised lymphadenopathy, fever, pharyngitis and skin rashes. Mortality is only 38% (Armitage et al., 1991), and spontaneous regression may occur. Lymphoma-like presentation is usually found in older patients, who often develop extranodal disease especially in the gut, central nervous system and the transplanted organ. Their response to therapy is poor and mortality is 71%. A diffuse polymorphic B cell hyperplasia develops mainly with the primary EBV, IM-like type of presentation while a B cell lymphoma tends to occur in the EBV-seropositive patients with extranodal disease. EBV serological testing for antibodies to the lytic cycle antigens (IgM and IgG to VCA and IgG to EA) is not diagnostic in LPD since the majority of immunosuppressed individuals show a ‘reactivated’ pattern with high levels of IgG antibodies to VCA and EA (Crawford and Thomas, 1993).

1.4.2.6 Non-Hodgkin’s lymphomas and other Malignancies

Non-Hodgkin’s lymphomas are a numerous, heterogeneous group of malignancies that originate from lymphocytes, they can develop from lymph nodes or other sites, many lymphoid neoplasms pass through both solid tumour and circulating (leukaemic) phases (IARC Monographs, 1997). Sinonasal T cell lymphoma is the most strongly associated with EBV, and the virus is highly prevalent in lymphomatoid granulomatosis. EBV is almost universally present in brain lymphomas secondary to immunodeficiency. The rate of detection of EBV is relatively low in B cell lymphomas of the nasopharynx, tonsil and tongue. Patients with X-linked lymphoproliferative syndrome are at increased risk for non-Hodgkin’s lymphoma, these patients have a specific defect in their control of infection of EBV. In one study EBV was found in all of 82 lymphomas associated with this condition (Sullivan and Woda, 1989). Several studies have indicated that EBV is associated with about 80% of lymphoepithelial gastric carcinomas (Rowlands et al., 1993, Osato and Imai, 1996). Smooth muscle tumours in immunosuppressed transplant recipients have frequently been found to contain EBV (McClain et al., 1995, Lee et al., 1995a).
1.5 Immunological Aspects of EBV-Related Disease

1.5.1 Serology of EBV Infection

Studies of IM patients have provided the bulk of the information available on primary antibody responses. The typical antibody pattern for primary EBV infection is characterised by IgM and IgG antibodies to virus capsid antigen (VCA), IgG antibodies to early antigens (EA) and the absence of antibodies to nuclear antigens (EBNA) (See figure 5).

![Antibody responses during EBV-induced infectious mononucleosis](image)

Figure 5: Antibody responses during EBV-induced infectious mononucleosis. (Adapted from Purtilo and Heinrich, INCSTAR Corp)

A gradual increase in anti-EBNA IgG antibodies occurs during convalescence and after four weeks VCA-IgM antibodies disappear. A possible explanation for the delayed antibody response to EBNA1, is that EBNA1 is protected from certain pathways of antigen processing. In adults also IgA anti-VCA arise frequently, whereas young children develop IgA only in 10-30% of the cases. Once infected, IgG antibodies to VCA and EBNA1 persist in healthy individuals for life. VCA-IgG is at a lower level than that at acute phase. Antibodies to EA-D (diffuse EA component) show a transient rise in the acute phase and are generally undetectable three to six months after onset. Anti-EA-R
(restricted EA component) antibodies are sometimes present in children younger than two years of age and in asymptotically infected patients IgM antibody to EA can reappear during EBV reactivation, there is also an increase in VCA-IgG and a decrease in EBNA1-IgG titres, in most cases of reactivation VCA-IgM is not detected The levels of each of these antibodies are generally lower in young patients, however, the profile does not differ with age The titres of antibody to each antigen and the time taken to develop a full spectrum of antibodies varies between patients See table 4

<table>
<thead>
<tr>
<th>Ab-Ag</th>
<th>Not infected</th>
<th>Silent primary infection</th>
<th>Past infection</th>
<th>Re-activation</th>
<th>IM</th>
<th>BL</th>
<th>NPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM-VCA</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IgG-VCA</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>IgA-VCA</td>
<td>-</td>
<td>-</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>IgG-EA/D</td>
<td>-</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>IgA-EA/D</td>
<td>-</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>IgG-EA/R</td>
<td>-</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>IgM-EBNA</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>IgG-EBNA</td>
<td>-</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>n/d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heterophile</td>
<td>-</td>
<td>−</td>
<td>+/−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

+ positive antibodies, ++ elevated antibodies, - negative antibodies, n/d no data

Table 4: Serological patterns of EBV antibodies in patients with EBV associated syndromes (Adapted from Lennette, 1991, Purtilo and Herrnchs, INCSTAR Corp)

Neutralising antibodies are detectable during the acute phase of IM but only at very low titres, which increase to stable levels thereafter (Horowitz et al., 1975, Lennette et al., 1982) Patients in the acute phase show IgG-response to EBNA2 and probably EBNA3A, 3B and 3C also Healthy virus carriers consistently have antibodies to VCA, neutralising anti-gp350 antibodies and antibodies to EBNA1 (Henle et al., 1987) Only a proportion of healthy carriers have antibodies to EA or to one or more of the other EBNA proteins There is an unusually strong cell-mediated immune response to clinically manifested primary EBV infection, which plays an important role in the control of persistent EBV infection A prospective study of IM patients showed that CTL memory increases during convalescence and soon reaches a stable steady state (Rickinson et al., 1980)
1.5.2 Historical Aspects of Serodiagnosis

Many years before the Epstein-Barr virus (EBV) was discovered serodiagnosis of infectious mononucleosis (IM) was based on the Paul-Bunnell test (Paul and Bunnell, 1932). In addition to EBV-specific antibodies, the early phase of IM is characterised by a general increase in total IgM, IgG and IgA, which is consistent with virus-driven polyclonal activation of the B cell system. The presence of heterophile antibodies in sera of IM patients was originally detected by agglutination of sheep, horse or bovine blood cells presenting heterophile antigens on their surfaces (heterophile antigens occur in unrelated species of animals but have similar serologic properties among them). This was later modified with the introduction of guinea pig kidney adsorption of serum to prevent interference of Forssman-type antibodies (Davidsohn, 1937). More recently, enzyme-linked immunosorbent assay (ELISA) techniques have been introduced, in which reaction tubes are coated with purified heterophile antigens. Heterophile antibodies are usually detected less than one week after onset of IM, peak at two-five weeks and can be detected, at low levels, a year after onset of illness (Niederman et al., 1968; Evans et al., 1975). This method is rapid and cost-effective and is still widespread in routine diagnosis, however, the test fails (10-15%) in detecting primary EBV-infection, particularly with young children (Lenette, 1991). Consequently serological assays have been introduced, these assays involve the measurement of the EBV-specific antibody response.

Primary EBV infections lead to the appearance of antibodies to specific EBV antigens which are usually detected by immunofluorescence (IF) staining of EBV infected cells (Henle et al., 1974). Primary infection may or may not be accompanied by clinical signs of IM. These IF assays are time consuming, difficult to evaluate, can’t be standardised exactly and may be hampered by autoantibodies to various cellular antigens which emerge in the course of IM in most patients (Sutton et al., 1974). Humoral responses to primary EBV infection are quite rapid; eighty percent of patients have peak titres when they consult their doctor. The type of infection a patient has can be determined, as the level and spectrum of antibodies are sufficiently distinct. The serological profile can indicate if the patient is still susceptible, has a current primary infection, has had a recent
primary infection (within 2-3 months), had a past infection or may have a reactivated EBV infection (Lennette, 1991).

1.5.3 Comparison of Commercially Available Diagnostic Kits
A number of studies have been undertaken to evaluate the specificity and sensitivity of the commercially available EBV diagnostic kits. A study undertaken in Germany evaluated eleven immunoassays for the detection of IgM antibodies to EBV (Weber et al., 1996). The kits evaluated were, Fresenius, BAG, Diamedix, Progen, Viro-Immuno, Virotech, Sigma Diagnostics, Viramed, Biotest, Behring and Savyon. An immunofluorescence assay was used as the standard as the symptomatology of IM may be attributable to other viral infections, i.e. HCMV and HIV. The IFA is the only test method for which antibody profiles have been correlated with clinical disease. (Wiedbrauk and Bassin, 1993). The results showed that there were great differences in the quality of current EBV-IgM-ELISA test kits. The Fresenius and Diamedix ELISAs showed the highest correlation with the reference IFA, the Biotest also correlated very well while the Virotech assay had very poor sensitivity.

Another study carried out in Sweden evaluated 6 kits that used purified heterophile antigen for the rapid diagnosis of IM compared with EBV-specific serology (Elgh and Linderholm, 1996). Three latex agglutination-based kits were involved, Monolatex, Mono-Latex and Mono-Lex, and three solid-phase-based kits, Mono-Plus, IM-Check and Clearview IM. Epstein-Barr virus-specific serologies including detection of VCA IgM and IgG and EBNA1 IgG, were used as a reference method. Five of the methods utilised purified bovine heterophile antigen, IM-Check was based on horse heterophile antigen. IM-Check was the only test which was not recommended for the confirmation of EBV-associated IM, the other five tests were recommended. Clearview IM combined a high sensitivity and specificity with a very simple one-step solid-phase-based procedure. This evaluation indicated that purified heterophile antigen-based rapid tests have higher specificity than the whole red blood cell agglutination assays for the diagnosis of EBV-induced IM. However, the heterophile antibodies go undetected in greater than 50% of children less than seven years old (De Ory, 1991).

A more recent study in Spain tested the reliability of new indirect tests in the diagnosis of acute EBV infection (Gutierrez et al., 1997). Mono-Latex, Enzygnost, Biotest
Diagnostics and the IgG avidity test were evaluated. The antibodies to antigen pools of virus (Enzygnost) had high reliability, the IgG avidity test is simple and automated in the laboratory, and is useful for determining the time since infection from a single sample. Mono-Latex and Biotest were shown to be lacking in sensitivity, specificity and the negative predictive value was low. This finding for Mono-Latex and Biotest does not correlate with the results from the previous two studies described. The reference method used and the range of sources of sera used may have implications in the differences in results found in both these studies.

In China a study was undertaken to evaluate multiple antibodies to EBV as markers for detecting patients with EBV-associated nasopharyngeal carcinoma (NPC) (Liu et al., 1997). Five serological tests were assessed for their sensitivity for screening and early detection of NPC, these included the detection of, (1) VCA using IFA, (2) EBV DNase using an activity neutralisation assay (NT), (3) EBV DNase using an ELISA, (4) DNA polymerase (DP) using a neutralisation assay, and (5) major DNA binding protein (MDBP) using an ELISA. It was found that no single test was sufficient for the detection of all NPC patients, however the detection of anti-DNase by ELISA was found to be the most sensitive method for detection of NPC.

1.5.4 Developments in Diagnosis

There is currently work being done to improve existing diagnostic techniques and to develop more sensitive and specific diagnostic methods. The detection of IgA antibodies to viral antigens is also being investigated. IgA is the principal antibody in mucous secretions, e.g. saliva, intestinal secretions and colostrum (Cooper, 1982). Studies in rats, mice and humans suggest that IgA is involved with transport of foreign antigens from the circulation into the bile (Russell et al., 1981). There have been many reports of detection of specific IgA antibodies in viral infections, for example, HSV and CMV (Hadar and Sarov, 1984, Sarov and Haikin, 1983).

In one case study anti-VCA IgA antibody was detected in the serum of almost 50% of IM patients screened using an immunoperoxidase assay (Hadar et al., 1995). The EBV-VCA-specific IgM antibodies were more sensitive (95-98%) and are more valuable for early diagnosis of IM, however, the IgA antibodies appear to be more useful as a marker.
for EBV reactivation. Serum-specific IgA antibodies to EBV-VCA have a high titre in most patients with nasopharyngeal carcinoma also. It has also been shown that EBV-VCA IgA antibodies in HIV-1 infected homosexual men may serve as an indicator for EBV reactivation, which seems to precede changes in the status of HIV-1 infection (Margalith et al., 1990). Similarly, other work has shown that the inclusion of the detection of IgG and IgA antibodies against EBV-VCA in a diagnostic test makes possible the diagnosis of chronic EBV infections and reactivations as well as primary infection by detection of IgM antibodies (Dopatka and Schuy, 1996). Anti-EA IgA is also known to be a highly specific marker for NPC (Henle and Henle, 1976). Work has been done on the development of an ELISA-based method for the detection of serum and saliva IgA against EA-D (Nadala et al., 1996). The results indicated that this method has potential as it was very specific, however, sensitivity was lower than that found with immunoperoxidase assay (IPA). Further work is being done to improve sensitivity without the loss of specificity.

An ELISA-based test has also been developed for the detection of IgG and IgM antibodies to three recombinant EBV antigens for routine diagnosis of EBV primary infection (Farber et al., 1993). The early antigens MDBP (truncated) and EA-D were used for detecting IgG and IgM antibodies, and EBNA1 (carboxy-half) was used for detecting IgG antibodies. These recombinant antigens have been proven effective for the standardised rapid diagnosis of acute EBV primary infection by ELISA, in comparison with the IF technique. Using highly purified recombinant EBV polypeptides as antigens may solve the problem of autoantigens encountered when using IF. A number of groups have investigated this possibility (Wolf et al., 1985, Motz et al., 1986, Hinderer et al., 1988, Gorgievski-Hrsoho et al., 1990).

The enzyme immunoassay has many potential benefits over the IFA test, including cost reduction, time-saving, and greater reproducibility. It is necessary to establish appropriate levels for determining a positive or negative result in comparison with the IFA test. It is possible to detect anti-EA antibody by EIA and to differentiate between the various EBNA components, with IFA this is not possible. It is also possible to detect IgM antibodies to EBNA which are not typically sought using the IFA test. The increased sensitivity provided by the EIA test allows the characterisation of individuals.
who are in transition from acute infection to latent or chronic infection. However, it may not be possible to correlate levels of antibody detected by IFA with those detected by the EIA, at all times. This is due to the amount of antigen used to coat the EIA wells as well as the selection of specific components of the viral proteins such as EBNA (Purtilo and Hinrichs, INCSTAR Corp).

ELISAs have been developed using recombinant EBNA1 truncated protein and a p18-VCA synthetic peptide. IgG antibodies are measured for the EBNA1 ELISA while both IgG and IgM antibodies are measured in the p18-VCA ELISA (Rosa et al., 1994, Liu et al., 1994). The VCA IgG test had a sensitivity of 98.2% and specificity of 99%, the VCA IgM test had a sensitivity of 96% and specificity of 98% in comparison to a commercial IFA. The EBNA1 IgG test showed a sensitivity of 85.9% and a specificity of 92.5% as compared to EBNA ACIF responses. The IgG responses to EBNA1 did not cross-react with CMV, HSV-1 and 2, VZV, Toxoplasma or Rheumatoid factor, also, the increase of EBNA1 peptide IgG responses was found to correlate well with the decrease of VCA IgM responses.

The replicative phase of the EBV life cycle seems to be restricted to epithelial cells of the nasopharynx, this results in release of viral particles in the saliva and the surrounding tissue (Greenspan et al., 1985, Young and Sixbey, 1988, Becker et al., 1991). The IgG response against the structural proteins of the virus, that is the major membrane protein and the capsid proteins MCP, p18 and p40, is initiated by encounter with local lymphoid tissue. In one particular study the antigenic domains of p18VCA were mapped (van Grunsven et al., 1994). This was done using the Pepscan technique (Geysen et al., 1984 and 1987), peptides of these domains were synthesised and their diagnostic relevance determined via ELISA. A single peptide was synthesised in which all the major epitopes of p18-VCA were combined, (peptide IV). Sera from healthy persons, and patients with IM and NPC whose diagnosis was previously determined using a heterophile antibody test were used in the evaluation.

Peptide IV showed improved reactivity with human antibodies in comparison with the individual peptides synthesised, and had a sensitivity of 95% for anti-VCA IgG and IgM. The specificity was determined using sera from EBV-negative blood donors who had
antibodies against other herpesviruses, all sera were proved negative for peptide IV. The IgG reactivity was detectable in sera from latently infected persons from the US, Hong-Kong, and the Netherlands, this shows worldwide conservation of the antibody response to the EBV-specific epitopes represented by VCA peptide IV. In general 80-90% of NPC patients have been shown to possess anti-VCA IgA antibodies, however, only 61% of the NPC sera tested contained anti-VCA peptide IV IgA antibodies. This peptide represents an important immunodominant EBV-specific reagent that may allow the production of a relatively cheap, reproducible EBV ELISA, however, it’s usefulness in NPC diagnosis is limited. Also sensitivity of 95% to IgG and IgM antibodies may not be sufficient, and additional EBV markers would be needed for 100% sensitivity.

1.6 Vaccine Development
In 1976 Epstein proposed a case for the development of an EBV vaccine (Epstein, 1976), since then a number of projects have been undertaken to develop and evaluate many different candidate vaccines. Killed, inactivated or attenuated virus cannot be used as the viral DNA is potentially oncogenic, and EBV is capable of sustaining a persistent lifelong latent infection. All vaccine candidates to date have been based on the gp350/220 subunit of the membrane antigen complex. This protein has been selected as it is the major envelope glycoprotein and is a major target of virus neutralising antibodies.

1.6.1 EBV Vaccine Candidate - gp350/220
The membrane antigen complex is involved in mediating virus binding to the B lymphocyte receptor CR2 (Nemerow et al., 1985; Tanner et al., 1987) and consists of at least four major EBV-induced glycoproteins, gp350/220, gp250/200, gp85, and gp78/55 (Mackett et al., 1990). During the acute phase of infectious mononucleosis there is an antibody response to gp85, however, there is none to gp350/220 (Henle et al., 1979), this finding is important as the antibody to this complex is the most potent source of neutralisation of the virus. Anti-gp350/220 monoclonal antibodies prevent virus infection and abolish it’s ability to transform B-cells. It has been suggested that antibody to gp350/220 binds to productively infected cells, making them susceptible to antibody-dependent cellular cytotoxicity (ADCC)-mediated lysis (Patarroyo et al., 1980). T cells play an important role in controlling EBV infection, recent studies have shown that T cells have a role in inhibiting cells expressing replicative antigens, including gp350/220
(Bejarano et al, 1988) If an antigen is found to induce antibodies which can both neutralise the virus and inhibit its binding to target cell receptors, this antigen or its active peptide would represent a preferred component of an effective EBV vaccine. For this reason efforts in the field of vaccine preparation against EBV have been directed primarily towards the use of these EBV-induced glycoproteins.

A study was carried out to evaluate the degree of conservation of gp350/220 between B95-8 (type A) and AG876 and P3HR-1 (type B) (Lees et al, 1993). Twenty four amino acid changes exist between the type A and type B strains, 9 of these cluster in the region of the protein which is spliced out of gp220. The gp350 molecule has a high proportion of serine, threonine, and proline residues, the majority of which are within the repeat region. This repeat region varies considerably in length and is the section removed from gp220. These facts suggest that this portion of the molecule is not required for persistence and replication of fully functional virus.

A number of B-cell epitopes have been identified on the gp350 molecule (Pither et al, 1992a,b) and three non-virus neutralising monoclonal antibody binding sites have been mapped by Zhang et al, 1991. Two virus-neutralising epitopes are known to exist but their location has not yet been identified (Qualtiere et al, 1987). It is thought that the virus-neutralising epitopes are discontinuous and conformation dependent, therefore native structure is required to elicit a neutralising serum response (Hoffman et al, 1980, North et al, 1982, Qualtiere et al, 1987). The monoclonal antibody 72A1 neutralises both B95-8 and P3HR-1 indicating that the epitope is present in both type A and type B viruses (Sairenji et al, 1988). This epitope is thought to be located within the amino-terminal 162 aa (Tanner et al, 1988). Two T cell specific epitopes have been mapped by Wallace et al, 1991, located between positions aa 61-81 and 163-183. A further study has confirmed the action of 72A1 with type A and B, and shown the conservation of several B cell epitopes within the gp350/220 molecule of both strains (Lees et al, 1993).

The variant loci within the EBV genome seem to be confined to the latent genes, while proteins associated with productive infection are more highly conserved. The high level of homology between EBV MA genes may be due to structural/functional constraints or possibly due to the fact that the virus latently infects B cells and transcription of most of...
the genome is consequently downregulated, thus reducing the immunologic selection pressure usually seen in other viruses. Type A is predominant in most parts of the world, however, both type A and B seem to have equal oncogenic potential in their association with BL and NPC (Young et al., 1987, Chen et al., 1992). Therefore, a vaccine based on the B95-8 gp350/220 should be equally effective against both types of EBV.

There appears to be species-specific variation in the immune response to EBV, as the epitopes found using serum from healthy seropositive humans were located toward the carboxy terminus of gp350 and were thought to be discontinuous in nature (Pither et al., 1992a), while those found after immunisation of rabbits were amino-terminal and continuous (Pither et al., 1992b). This difference in response may also depend on whether the individual is naturally infected or vaccinated. The molecule’s host cell-specific pattern of glycosylation will also influence the accessibility and structure of the gp350/220 molecule.

1.6.2 Neutralising Antibodies to EBV

Eight monoclonal antibodies have been identified which are capable of neutralising EBV strain B95-8 (Qualtiere et al., 1987), one of these 72A1, an IgG2a class antibody, has also been shown to neutralise type B strain P3HR-1 (Sairenji et al., 1988), both with and without complement. This antibody completely eliminated infectious virus from supernatants of B95-8 cells, however, in the case of P3HR-1, 72A1 never completely eliminated infectious virus from the supernatants, even at high MAb concentrations, but decreased the virus titre from 1/10 to 1/50. B95-8 EBV possesses larger amounts of gp350/300 than gp250/200 on its surface, while gp250/200 is more abundant on P3HR-1 than gp350/300 (Edson and Thorley-Lawson, 1981, North et al., 1980). This may somewhat explain the difference in the effect of 72A1 on the different strains. A previous study showed that of eight monoclonal antibodies analysed only four were capable of neutralising EBV and one of these four (72A1) could inhibit EBV binding to its target receptor (ie CR2). It is probable that the epitope recognised by 72A1 is the only glycoprotein domain with a dual role in EBV neutralisation and binding to target cell receptors (Stocco et al., 1990). If the epitope recognised by 72A1 could be identified it
may be possible to develop a vaccine based on this peptide sequence which would both neutralise the virus and simultaneously prevent binding of the virus to its target receptor.

The third component of the MA gp85 is encoded by the BXLF2 ORF, this is an 85kD glycoprotein found on the envelope of the virion, as well as the surface of EBV-producing cells. F21 is an IgG2a class antibody against gp85, which can neutralise EBV only in the presence of complement. gp85 appears to play an important role in the fusion of virus envelope to the target cell membrane (Miller and Hutt-Fletcher, 1988). F21 inhibits this fusion but cannot inhibit binding of the virus to its target receptor. G31 another anti-gp85 IgG2a antibody can also neutralise the virus and inhibit the fusion process. The inability of these antibodies to prevent virus attachment indicates that the epitopes they recognise play no role in EBV binding.

1.6.3 The Cytotoxic T Cell Response to EBV Infection

The study of other viral systems has shown that there exists a central role for CD4+ T cells in the induction of specific immune responses to vaccine antigens, this activity has the potential to enhance both antibody responses and CD8+ cytotoxic T cell responses to a subsequent natural infection (Wallace et al., 1991). CD4+ T cells recognise foreign antigens as small peptides which are generated by processing within antigen-presenting cells and are presented at the cell surface as a complex with MHC class II molecules (Schwartz, 1985). The CD4+ T cell responses of individuals to EBV-vaccination have been investigated (Wallace et al., 1991). The locations of two epitopes were found, as described earlier. Identifying these epitopes within the primary sequence could facilitate effective vaccine design. T cell clones with different epitope specificities were found to be restricted through different HLA class II antigens, therefore it will be necessary to screen T cell clones from a greater number of donors to assess the influence of HLA class II polymorphism upon epitope choice. To identify an epitope capable of forming complexes with several polymorphic HLA-DR molecules would be desirable, as has been found in other systems such as HIV and malaria (Singaglia et al., 1988, Berzofsky et al., 1989).
In order to study the Epstein-Barr virus a number of animal models have been developed. Since the early eighties many vaccine trials have been undertaken, in which these animal models have played an essential role. Mice, rabbits, common marmosets and cottontop tamarins have been involved in such trials. Cottontop tamarins when challenged with a large dose of EBV develop multiple B cell lymphomas within 2-3 weeks, these lymphoma cells contain the EBV genome (Cleary et al., 1985). The tumours are progressive and fatal in most cases, but a number of animals survive, the tumours regress in these individuals over a period of 8-12 weeks. They are completely immune to a second challenge with EBV (Finerty et al., 1988).

Human and tamarin EBV infections differ in a number of ways, however, in vivo the B cell tumours resemble those in immunosuppressed humans. It is also possible to establish EBV-transformed lymphoblastoid cell lines (LCLs) from tamarin peripheral blood B cells (Miller et al., 1972). EBV does not establish a lifelong latent infection of tamarins at the same level that occurs in humans (Finerty et al., 1988, Niedobitek et al., 1994), and the dose of virus is 50,000 times that estimated to transfer infection between humans. An infectious mononucleosis-type disease is not established in the tamarin model and it has not been possible to infect them via the mucosal route. This raises the question as to whether this is a suitable model for vaccine development, however, the immune responses of both the tamarins and humans are similar, each have an effective cellular immune response (Finerty et al., 1988, Rickinson et al., 1989). Despite the fact that the cottontop tamarin is less than the ideal model for human infection and disease, the induction of lymphoma by a large dose of virus is completely reproducible and could be considered as representing a 'worst-case' infection and in this respect is a useful measure of the efficacy of any experimental vaccination (Morgan, 1992).

From previous vaccination trials in tamarins it appears that a cell-mediated immune response is essential for protection against further EBV challenge. When immunised with a gp350 envelope glycoprotein in liposomes the tamarins developed high neutralising antibody titres but were not protected, however immunisation with a recombinant
vaccinia virus protected them from EBV challenge in the absence of a neutralising antibody response (Epstein et al., 1986; Morgan et al., 1988). Similarly, a replication-deficient recombinant adenovirus (Ad) used to immunise cottontop tamarins, protected animals against further viral challenge without the development of neutralising antibodies (Ragot et al., 1993).

1.6.5 Vaccinia Virus - a Vaccine Vector

There have been developments in potential new vaccines based on the expression of foreign genes in vaccinia virus, hepatitis B surface antigen, herpes simplex virus type 1 glycoprotein and influenza virus haemaglutinin (Smith et al., 1983; Moss et al., 1984; Paoletti et al., 1984). Vaccinia virus was used successfully in the eradication of smallpox world wide, these results prompted the development of a recombinant vaccinia virus (Wyeth strain, New York city board of health) expressing EBV gp350 (Mackett and Arrand, 1985). The protein possessed the properties of native gp350, it was heavily glycosylated and could be detected at the surface of infected cells. When used to vaccinate rabbits, they produced antibodies that recognised EBV-containing lymphoblastoid cells and neutralised EBV. In the more recent study by Morgan et al., 1988, a second recombinant vaccinia virus (WR laboratory strain) was constructed again expressing EBV gp350. In this case protection against EBV-induced lymphoma in cottontop tamarins was obtained with the WR strain but not with the Wyeth strain. This difference was thought to be due to the thymidine kinase (TK) negative phenotype of the Wyeth strain. A TK-negative virus (vTK-16) was used to produce a gp350 recombinant vaccinia virus in the vaccination of common marmosets (Mackett et al., 1996). The gp350 gene used was from the M81 strain, which is closer to the wild type strain of EBV circulating in the general population than the widely used B95-8 strain. Infection of the common marmoset with the M81 strain is followed by responses similar to those commonly seen in man, ie. lymphocytosis, heterophile antibody and longterm production of antibody to viral antigens (de Thé et al., 1980; Wedderburn et al., 1984). The long-term EBV-host interactions are established essentially without clinical disease, similar to natural infection in the majority of cases. Successful vaccination was reported, that is, there was a decrease in virus load compared to controls after oral challenge with EBV. Vaccination does not prevent infection but can prevent clinical disease. Further work is
necessary to improve the safety of vaccination with vaccinia virus for human trials, without compromising the efficacy of the vaccine. The first EBV vaccine trial in humans using recombinant vaccinia virus was carried out in 1995, the entire gp350/220 gene was expressed and resulted in protection against and/or delay of EBV infection by the natural route in humans (Gu et al., 1995).

1.6.6 Adenovirus - a Vaccine Vector

Human adenoviruses have considerable potential as expression vectors and live-virus vaccines for a wide variety of human and animal diseases. Ad 5 recombinants carrying inserts of HSV, VSV or rabies glycoprotein-coding sequences have been shown to raise neutralising antibodies in rhesus monkeys, cows, pigs, dogs, foxes, striped skunks, raccons, and mice (Graham and Prevec, 1992). The first human use of an Ad vaccine vector expressing HBsAg was undertaken by Tacket et al., 1992 in a phase I study. Ragot et al., 1993 have developed a recombinant adenovirus serotype 5 expressing the full length gp350/220. The gp350/220 gene was cloned into the E1A region of the Ad genome which encodes the immediate early proteins that trans-activate all other early regions, therefore making the mutant replication-defective. It has been shown that even without replication such vaccine vectors can induce good humoral immune responses in animals (Ballay et al., 1985; Alkhatib and Briedis, 1988; Eloit et al., 1990; Ragot et al., 1991; Levrero et al., 1991). Recombinant Ad constructs expressing gp350/220 have been shown to induce anti-gp350/220 antibodies in rabbits and their sera was EBV-neutralising in vitro (Ragot et al., 1990). In this study the ability of one of these constructs to protect cottontop tamarins against EBV-induced lymphomas was investigated (Ragot et al., 1993). The tamarins were completely protected following intramuscular injection, immunisation via the oral route is being investigated as the mode of immunisation may result in a different type of immune response. The sera obtained from the protected tamarins following intramuscular introduction were not virus-neutralising. This reinforces evidence that the cellular immune response plays an important role in protection. Previous work has shown the absence of correlation between neutralising antibody and protection (Epstein and Achong, 1986). It is not necessary for neutralising antibodies to be produced following immunisation in order to develop protective immunity. The use of recombinant live viruses as vaccine vectors has
many advantages, effective T cell antigen presentation, absence of the need for adjuvants and low production costs. There is the risk of horizontal transmission between humans or between humans and other animal species, however, a replication-defective Ad vector greatly increases the safety.

1.6.7 Varicella-Zoster - a Vaccine Vector

An attenuated strain of varicella-zoster virus (Oka) has been developed for use as a live viral vaccine, it has been clinically tested and proven to be both well tolerated and effective in preventing chicken pox (Takahashi et al., 1974). A recombinant varicella zoster virus has been produced which expresses the EBV glycoprotein gp350/220 (Lowe et al., 1987). Varicella-zoster has been shown to be safe and efficacious in vaccination of immunocompromised individuals (Feldman et al., 1973), an important consideration for EBV immunisation. This reinforces the potential of this recombinant for use in EBV vaccination trials.

1.6.8 Subunit Vaccines

The advantages of using live virus vectors are, the induction of broad ranging immune responses, including long term immunological memory, relatively low production costs and the potential for use as single dosage oral vaccines. There are also a number of disadvantages such as possible reversion to pathogenic forms by mutation or recombination with wild types, possible alternative host cell tropism and poorly defined immunogenic activities. Development of a subunit vaccine could overcome a number of these disadvantages. A subunit vaccine is clearly defined and a pure product which contains no genomic material or replicative capacity. These factors are important when considering health and safety regulations. The need to administer a subunit vaccine with adjuvant does pose a problem. The only adjuvant licensed for general human use is alum. Alum is a relatively weak adjuvant and is defective in its ability to induce appropriate cell-mediated immune responses. Alternatives have been explored and found effective, these include muramyl dipeptide derivatives administered in synthetic oils (Byars et al., 1991), and immunostimulating complexes (ISCOMS) (Morein et al., 1984). These adjuvants have been tested in the tamarin model and were found to be excellent alternatives to alum (Morgan, 1992). An appropriate adjuvant must be selected before
subunit vaccine trials can be undertaken in humans. Substantial amounts of gp350 can now be produced in conditions compatible with good manufacturing practice and in the certain absence of any potentially oncogenic EBV DNA. Biodegradable microparticles as controlled antigen delivery systems are an attractive alternative to alum or other adjuvants but have yet to be tested in the tamarin lymphoma model (O’Hagan et al., 1991). The identification of the neutralising epitopes on the gp350/220 molecule, and the subsequent synthetic production of these peptides could provide an alternative subunit vaccine. The production of recombinant gp350/220 would be eliminated and a number of peptides, possibly from the latent antigens of EBV, could be incorporated in order to induce a broad immunological response, T cell epitopes could also be included in this type of vaccine.

1.6.9 Vaccines Based on Peptide Epitopes for CTLs

Cytotoxic T-lymphocyte (CTL)-mediated immunity is a specific and potent response to EBV infection. The CTL response is directed primarily against latently-infected B cells, with EBNA3A, 3B and 3C being the major targets (Murray et al., 1992; Khanna et al., 1992). The possibility of priming the T cells by peptide immunisation before infection occurs could accelerate the CTL response to primary infection and thus limit subsequent colonisation of the B cell pool. This strategy would involve the use of synthetic peptides representing the major CTL target epitopes presented by common HLA alleles (Rickinson, 1995). A large number of epitopes have now been identified and calculations have shown that a polytope vaccine containing only 7 epitopes restricted by HLA A2, A3, A11, B7, B8, B35 and B44 would protect >90% of a Caucasian population (Suhrbier, 1997). The chance of developing IM after EBV infection is only 50% in adolescents, therefore, a few EBV-specific memory CTL might be sufficient to tip the balance to asymptomatic seroconversion. EBV vaccines based on CTL induction would reduce morbidity rather than prevent infection. It has been shown that only a small number of adoptively transferred EBV-specific CTL were sufficient to clear an LPD that was X-ray-visible (Rooney et al., 1995).

EBV infection has been shown to result in significant morbidity and mortality in specific high risk populations and the extent of it’s impact remains undetermined. Despite the considerable cost involved in vaccination trials and a vaccination program, it appears that
an EBV-vaccine would be cost-effective. EBV has been incriminated in the pathogenesis of various malignancies, almost all cases of NPC, 95% of African BL, ~33% of BL in the United States, ~50% of HD, 11% of spontaneous non-Hodgkin’s lymphomas and about 33% of non-Hodgkin’s lymphomas in AIDS patients (Evans, 1993). The development of an EBV vaccine to prevent IM is considered cost effective and taking into consideration the malignant consequences of an EBV infection further reinforces the need for the development of such a vaccine.
Expression of EBV recombinant proteins in *E. coli* is the main part of the subject matter of this thesis.

Production of recombinant proteins on an industrial scale is essential due to many factors. The natural supply of a particular protein is too limited to allow widespread use. This is especially true for proteins used in the pharmaceutical industry, for example insulin, factor VIII, and growth factors. In some cases the supply exists but it is difficult to exploit, or may become limited as demand increases. It is also important when using proteins in pharmaceuticals or food production, that the products are as safe as possible, the absence of pathogens is crucial. If a particular protein is to be used in diagnosis it is important that contaminating proteins be removed to eliminate the possibility of cross-reactivity, and reduction of specificity.

Many hosts for production of heterologous proteins have been exploited, these include mammalian cells, insect cells, filamentous fungi, yeast and bacteria (both gram-negative and gram-positive). Mammalian cells are used extensively for the production of human proteins, the products retain their native structure and glycosylation is possible. In some cases this is important for correct protein function. Yeast cells also provide the advantage of allowing correct folding and processing of mammalian proteins. Cell culture is expensive, yield can be too low, and there may be problems with cross-reactivity if the products are needed in diagnosis. For this reason use of a bacterial host can be an advantage. *Escherichia coli* remains the organism of first choice for expression of heterologous genes as a result of it's ease of manipulation and the quantity of protein obtainable. *E. coli* is genetically and physiologically well defined, it is easily manipulated and high-level expression of many heterologous proteins has been achieved. It has also proven to be easily scaled-up from shake flask to large-scale culture and commercial production. However, with high-level production often product quality is sacrificed, recent advances in heterologous gene expression now allows, in many cases, enhanced protein quality in vivo and in vitro (Olins and Lee, 1993). The use of fusion proteins can aid maintenance of the correct secondary and tertiary structure of a particular protein as well as aid in protein purification. GroESL, DnaK, protein disulphide isomerase, and
prolyl isomerase used singly or in combination may prove useful in enhancing either the rate or extent of protein folding (Buchner et al., 1992, Freskgard et al., 1992). Fusion of cytoplasmically expressed heterologous proteins to other proteins can increase their solubility and biological activity (Smith, 1992, McCoy and LaVallie, 1992). Thioredoxin fusions allow a variety of mammalian proteins that would otherwise form inclusion bodies (IBs) to be expressed in an active and soluble form, which can be recovered by osmotic shock, or heat treatment (LaVallie et al., 1993).

*Escherichia coli* expressed recombinant antigens have been used in the development of ELISA-based assays for improved diagnosis of virus-associated diseases, two examples are hanta virus and dengue virus. In the case of Hanta virus, truncated recombinant nucleocapsid protein of five viral serotypes have been expressed in *E. coli*. These proteins were purified and successfully screened with sera from patients of each serotype for IgM and IgG reactivity (Elgh et al., 1997). An indirect ELISA has also been developed for diagnosis of dengue virus using a recombinant viral envelope B protein, which is expressed as a trpE fusion in *E. coli*. This antigen was found to be of equal sensitivity as cell culture-derived antigen for both IgM and IgG detection (Simmons et al., 1998).

1.7.1 The Thiofusion Expression System

The Thiofusion™ Expression System developed by INVITROGEN allows expression and purification of large amounts of heterologous protein from *E. coli*. The gene of interest is cloned into the multiple cloning site of the pTrxFus cloning vector, and are consequently expressed as fusions with the *E. coli* thioredoxin protein (trxA) (Bayer, 1968, Holmgren, 1985). Thioredoxin appears to confer solubility to formerly insoluble heterologous proteins, thus avoiding the problem of inclusion bodies (LaVallie et al., 1992). Fusion with thioredoxin may confer a unique cellular location allowing easy purification by osmotic shock, also the fusion protein may be heat stable allowing purification by heat treatment. In addition these fusions express at high levels (Lunn et al., 1982 and 1984). The P_L promoter from bacteriophage λ is used to drive expression in pTrxFus. This is one of the most efficient promoters for bacterial expression and is tightly regulated (Buell and Panayotos, 1986). The bacteriophage λ cl repressor binds to the operator region in front of the P_L promoter and controls the level of transcription from the promoter. The cl repressor expression is regulated by the trp promoter in the
*E. coli* cells (either G1698 or G1724) by trp repressor and attenuation. When cells are grown in tryptophan-free medium, the *cl* repressor gene is transcribed, and the *cl* repressor protein binds to the *P_L* promoter preventing transcription. Expression is induced by adding tryptophan to the medium, thus shuts down *cl* repressor synthesis, thus allowing transcription from the *P_L* promoter. Figure 6 shows a schematic diagram of the pTrxFus plasmid used in the cloning procedure.

**Figure 6:** Schematic representation of the pTrxFus plasmid.

- ampicillin ORF: bases 201-1061
- ColE1 origin: bases ~1300-1880
- *P_L* promoter: bases 2159-2187
- ribosome binding site: bases 2709-2726
- thioredoxin ORF: bases 2724-3050
- enterokinase cleavage site: bases 3051-3080
- multiple cloning site: bases 3081-3112
- aspA transcription terminator: bases 3113-3179

Expression of thioredoxin fusions is optimised by varying the growth temperature during induction to get the maximum amount of soluble fusion protein. The expression...
temperature can range from 15°C to 37°C for thioredoxin fusion proteins (Holmgren, 1985). There are two host strains, G1698 for expression of fusions below 30°C, and G1724 for expression at or above 30°C. They differ in their expression of the cl repressor. The G1698 strain has no ribosome binding site before the cl repressor gene, this causes decreased production of cl repressor even in the absence of tryptophan. G1724 has a good ribosome binding site leading to increased production of cl repressor relative to G1698, therefore, regulation is quite tight in G1724 and leaky in G1698. Since expression at lower temperatures may increase the solubility of the fusion protein, leaky expression may allow more of this soluble fusion protein to be expressed at lower temperatures.

Thioredoxin fusion proteins may localise to the adhesion zones (See Figure 7) (Lunn et al., 1984), where native thioredoxin normally localises. Adhesion zones are sites on the cytoplasmic side of the inner membrane. If fusions are located at this position they could be purified by osmotic shock (Lunn et al., 1982). Thioredoxin fusions may also be heat stable, and since not all will localize to adhesion zones, heat treatment can provide an alternative purification method. Most proteins precipitate after 1 minute at 80°C, while some thioredoxin fusions are stable for 10 mins at this temperature. There is an enterokinase cleavage site (Asp-Asp-Asp-Asp-Lys-Xaa) engineered into pTrxFus in a 30bp spacer between the TrxA gene and the multiple cloning site. Enterokinase hydrolyses the peptide bond between lysine and the next amino acid, except if the next amino acid is proline. The fusion protein must be partially purified for enterokinase to work efficiently.
Mouse brain fascin has been cloned and expressed in this thioredoxin expression system, producing mouse fascin as a soluble fusion protein (Edwards et al., 1995). Previous study of recombinant fascins was difficult, bacterially expressed fascins were insoluble, fusion with thioredoxin overcame this problem. The thioredoxin moiety was cleaved using \( \alpha \)-chymotrypsin, as fascin is relatively resistant to digestion to \( \alpha \)-chymotrypsin.

Seryl-tRNA synthetase from the extreme halophile *Haloarcula marismortui* has also been cloned into the pTrxFus plasmid for expression in *E.coli* (Taupin et al., 1997). Soluble active recombinant proteins were expressed however the activity level was poor.

1.8 Random Phage Peptide Libraries (RPLs)

Screening of random phage peptide libraries is the second part of the subject matter of this thesis.

It was first suggested by Dulbecco in 1982, that immunogenic peptides from well-characterised epitopes of pathogenic agents could be fused to the coat proteins of bacteriophage. The foreign peptides would be displayed on the surface of the viral particles and could be used as the principal components of cell-free vaccines (Dulbecco,
By 1985 it had shown that filamentous phage could easily be manipulated to produce phage displaying 'phagotopes' on their surface (Smith, 1985). Random peptide libraries (RPLs) can be used to survey tens of millions of short peptides for tight binding to an antibody, a receptor or other binding protein. The library consists of a huge mixture of filamentous phage clones, each displaying one peptide sequence on the virion surface. Fusion phage are filamentous bacteriophage vectors in which foreign antigenic determinants are cloned into phage gene III or gene VIII and displayed as part of the product of either gene.

Both chemical and biological libraries have been developed. The first chemical libraries were linear peptides displayed on glass beads or plastic pins, biological libraries include peptide display on surface proteins of E.coli (Little et al., 1993) the ‘peptides on plasmids’ approach, and phage display of peptides. In biological approaches, protein and peptide variants are expressed physically linked in some way to the genes that encode them, therefore, any selection procedure enriching for a particular mutant co-selects for it’s DNA sequence. Biological libraries can be endlessly regenerated unlike chemical synthetic libraries, however, they are subject to selective pressures independent of those imposed in vitro.

1.8.1 Structure and Biology of fd and M13

The filamentous phage infection cycle is initiated by the attachment of pIII to the tip of the F pilus of the bacteria, followed by internalisation of the single-stranded viral DNA (ssDNA). This ‘plus’ strand serves as template for minus-strand synthesis which results in a double-stranded replicative form (RF). The RF is the template for mRNA transcription, RF replication and production of ssDNA progeny. Progeny virions are assembled by extrusion of ssDNA through the bacterial envelope without killing the cell or preventing cell division. As it emerges from the cell, the ssDNA acquires it’s extracellular sheath of coat proteins from the membrane (Smith and Scott, 1993). The pIII protein is a 406-amino-acid protein synthesised with an 18-amino-acid leader sequence, which is later cleaved off. The protein has two domains of approximately equal size; in the viral capsid, the N-terminal region protrudes outwards, while the C-terminal domain is buried in the particle.
1.8.2 Peptide Fusions

Filamentous phage are the preferred vectors for the construction of 'genetic' repertoires for the following reasons: Insertions of foreign sequences at the amino terminus of either the pIII or the pVIII coat protein are, in general, well tolerated. The foreign sequence is displayed on the surface of the viral particle (See figure 8) and can bind to molecules such as antibodies and receptors (Smith, 1985). Sequences that interfere with the life cycle of the virus can be displayed by using the 'two genes' or 'phagemid' system, where chimaeric phage bearing both wildtype and recombinant coat proteins are produced. In a two-gene system, the fusion protein is supplemented with the corresponding wild-type protein, so that the particles bear a mixture of coat proteins, only some displaying the foreign domain. This system allows fusions that partially impair coat-protein function, and for that reason would not be tolerated in one gene systems. A phagemid is a plasmid containing the intergenic region of filamentous phage in addition to a conventional replication origin and an antibiotic resistance gene. The intergenic region contains all the cis-acting elements that form the site of initiation of replication and the morphogenetic signal, which are required for ssDNA synthesis and packaging into virions. When a cell with a phagemid is superinfected with helper phage that supply all the phage proteins, it secretes not only helper phage but also infectious phagemid virions (Smith, 1991).
The pIII protein from gene III is present in five copies at one end of the phage particle coat, while the pVIII protein from gene VIII has approximately 2700 copies on the surface of the phage (Scott and Smith 1990). The pIII minor capsid protein is used most frequently for display of peptides. This protein is required for infectivity, and can be seen under the electron microscope (EM) as protruding knob-like structures. pIII also stabilises the phage particle against dissociating agents (Hill and Stockley, 1996).

The major limitation in the use of pIII to present peptide sequences is the number of fusion proteins that can be displayed on each particle, a maximum of five. This may be an advantage in selection of peptides which have very high affinity for the ‘selector’ molecule as opposed to those with low affinity binding. Peptides up to a few hundred residues in length can be displayed on pIII (Smith, 1991). Alternatively, pVIII can be used for cloning as there is the potential to present more than 2700 copies of a peptide on each phage particle. The pVIII is a 50-amino-acid protein with a cleavable 23-amino-acid signal sequence. The first four or five N-terminal amino acids are found at the surface of the particle and it is in this region that insertions have been made.
for insertion into pVIII are more stringent than for pIII, and only 8-9 amino acids in length can be tolerated if present on every pVIII subunit of the capsid (Greenwood et al., 1991) (See figure 9)

![Figure 9: Classification of phage display vectors. (Adapted from Smith, 1993)](image)

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>a single recombinant gene III bearing a foreign peptide insert</td>
</tr>
<tr>
<td>33</td>
<td>2 genes III, one wild type the other an insert bearing recombinant</td>
</tr>
<tr>
<td>3+3</td>
<td>2 genes III, one wild type the recombinant gene is on a phagemid</td>
</tr>
<tr>
<td>8</td>
<td>a single recombinant gene VIII bearing a foreign peptide insert</td>
</tr>
<tr>
<td>88</td>
<td>2 genes VIII, one wild type the other an insert bearing recombinant</td>
</tr>
<tr>
<td>8+8</td>
<td>2 genes VIII, one wild type the recombinant gene is on a phagemid</td>
</tr>
</tbody>
</table>

It is possible to select the binding protein or peptides, of a particular ‘selector’ molecule, from a mixture of >10⁹ different phage particles (Cortese et al., 1994). This technique, commonly known as biopanning, was first described in 1985 (Smith, 1985), and has since been altered and modified for many different applications (See figure 10 for a schematic diagram of this procedure). The original method involved covalent linkage of biotin to the target molecule, this is done prior to incubation with the phage library. Phage able to bind the target are then selected by ‘panning’ the mixture of phage and
target molecule on a streptavidin-coated dish. This exploits the strong biotin-streptavidin interaction, retaining the target molecule and consequently the phage bound to it. Bound phage are then eluted from the complexes and subjected to further rounds of amplification in *E. coli* and biopanning. Usually three rounds of biopanning are necessary to isolate binding phage, on subsequent rounds a limited amount of target can be used to achieve competition between different clones, this facilitates the isolation of higher-affinity peptides. Individual clones must be independently assayed, because false positives may occur by the selection of clones that bind the support matrices, or streptavidin (Adey et al, 1995). A subtraction step may be used to avoid false positives, this involves panning the phage selected from the first round on a streptavidin coated plate in the absence of target molecule. This should, in theory remove those phage which are not specifically selected for by the target molecule.
Repeat twice

1. Coat plate with streptavidin
2. Immobilise biotinylated antibody on streptavidin coated plate
3. Add phage to the plate
4. Wash away unbound phage
5. Elute bound phage

Titer eluate and amplify in *E. coli* K91Kan

Figure 10: Flow diagram of the biopanning procedure.

### 1.8.3 Phage-derived Peptides and their Applications

The first phage display libraries polyvalently displayed random linear peptides with 6 (Scott and Smith, 1990, Cwirla *et al.*, 1990) to 38 (Kay *et al.*, 1993) residues being randomised. Libraries with longer random regions are incomplete due to the huge numbers of individual clones necessary, with several shorter random peptides contained within each clone, and may be useful if binding preferences are being sought as opposed to an optimum sequence. Longer peptides may be capable of assuming a wide variety of secondary structure motifs including α-helices, β-turns and mimics of antigenic loops on...
the surface of different proteins. A 30 mer library created by Grhalde et al, 1995, without any special constraints, contained a significant percentage of sequences carrying two or more Cys residues, and could present 'looped peptides' containing a disulphide bridge. Libraries that are theoretically complete may lack clones that are toxic to the host, disrupt protein folding, are proteolytically labile, or are poorly translated, secreted and/or processed (Clackson and Wells, 1994)

1.8.3.1 Epitope Mapping

Classical epitope mapping required a set of overlapping synthetic peptides spanning a region of the antigen, or a set of deletions obtained by genetic manipulation of the antigen-coding gene. If a peptide was found to react with the antibody, the epitope was declared to be linear or continuous, however, if a peptide could not be identified, the epitope was said to be discontinuous. A discontinuous epitope is one which is made up of amino acids closely related in space but distant in the primary sequence. This was an oversimplification of the actual situation and it is thought that 'complete' epitopes (i.e., all amino acids that make contact with the antigen-binding site) are probably all discontinuous to a certain degree.

The primary application of phage display libraries has been the identification of B cell epitopes and to a lesser extent T cell epitopes. There has been much success with linear epitopes, however, discontinuous epitopes have proven less amenable to this technology. An example of the success in identification of an epitope is a study with HIV. A monoclonal antibody 447-52D raised against the HIV-1 glycoprotein 120 V3 loop has been shown to neutralise the virus, as a result this protein (gp120) has been the focus of vaccine studies. A 15-mer epitope library was screened with the MAb 447-52D, the consensus sequence GPXR was identified in 98% of the isolated clones (Keller et al., 1993). The core of the recognised epitope is linear and this is thought to have contributed to the success of this study. An immunogen was created using one of the selected peptides and it was found to elicit an antibody response which neutralised two related viral variants. A later study focused on the isolation of peptide binders to anti-HCV (14-153-462 MAb) and anti-gp120 V3 loop (1001 MAb) (Gnhalde et al., 1995)
Both antibodies detect their respective antigens in western blot suggesting they bind linear epitopes. A 30 mer phage library was screened with both antibodies, the anti-gp120 antibody selected clones all contained a G-R sequence flanked by two cys residues. The presence of a disulphide bridge at the base of the loop constrains the peptide in a β-turn allowing binding of the antibody (Ghiara et al., 1994). This is consistent with the other findings of Keller et al., 1993. The G-R dipeptide is known to be part of the highly conserved G-P-G-R-A-F sequence present in many HIV isolates. Forty clones derived from selection against the HCV Ab were isolated and their sequence deduced, 22 unique sequences with the consensus R-X-R-R-Q-P were identified, which maps to the sequence R-G-R-R-Q-P at aa 59-94 of the HCV core protein sequence. These results illustrate the accuracy with which epitopes can be identified via phage display libraries.

Phage borne mimotopes have been isolated that mimic non-peptidic epitopes, two groups have isolated phagotopes that react with streptavidin, these molecules are likely to be analogues or mimics of biotin, on the basis of competition experiments (Devlin et al., 1990, Kay et al., 1993). Most of the phagotopes isolated display the consensus sequence HPQ, and appear to use only a subset of the biotin interaction sites at the binding pocket. Concanavalin A and the carbohydrate binding antibody B3 have also been used as selector molecules with phage libraries. The lectin Con A naturally binds carbohydrates, the common consensus sequence isolated for this protein is YPY (Scott et al., 1992, Oldenburg et al., 1992). The B3 monoclonal antibody which binds the carbohydrate antigen Lewis\(^\dagger\), selects for the consensus sequence PWLY (Hoess et al., 1993). A recent study involved the analysis of closely related mAbs directed against the cell-wall polysaccharide of group A Streptococcus (Harris et al., 1997). All of the antibodies isolated peptides which cross-react, from a panel of phage-displayed libraries. Competition studies indicated that many of the peptides bind at or near the carbohydrate binding site. The peptides were found to bind the mAbs by different mechanisms than the branched trisaccharide epitope, which is the epitope bound by each of the antibodies \textit{in vivo}. It has been suggested that the CHO-crossreactivity of Abs produced against CHO-mimic peptides is determined by factors other than their structural similarity to the antigen (Harris et al., 1997). Previous work has shown that in general the majority of
anti-CHO Abs use a limited subset of \( V \) genes, indicating that this class of Abs uses a restricted structural repertoire (Vargas-Madrazo et al., 1995) Other peptide mimics for non-protein ligands have also been identified, for example, anti-biotin PCAbs (Roberts et al., 1993), and iron oxide (Brown, 1992) In the case of iron oxide the peptides were displayed on the surface of bacteria

1.8.3.2 Antibody (Fab) Libraries

Libraries have also been created displaying antibody Fab fragments, the method used is known as ‘the combinatorial strategy’ (Huse et al., 1989) This involves the random recombination of light- and heavy-chain fragments (Fabs) expressed in phage \( \lambda \). Most work in this area is directed toward the cloning of human antibodies as these are the most difficult to obtain by other methods and are of therapeutic value Use of phage display allows the production of antibodies without immunisation Human antibodies have been cloned that bind HIV (Barbas, CF III et al., 1992a), hepatitis B (Zebedee et al., 1992), respiratory syncytial virus (RSV) (Barbas, CF III et al., 1992b), cytomegalovirus, herpes simplex virus types 1 and 2, varicella zoster virus, measles, and rubella Single chain antibodies have also been displayed on phage, however, the single chain antibodies that have been selected have been found to have very low affinity, and only those which dimerise on the phage and subsequently in solution are selected (Griffiths et al., 1993) Using a helper phage which is deleted in pIII converts a monovalent phagemid system into a multivalent system, aiding the capture of these low affinity clones Phage libraries displaying Fab fragments which are monomeric, have proven more effective than other antibody libraries in the isolation of high-affinity clones

1.8.3.3 Phage Display of Protein Ligands

In theory, any protein secretable to the periplasmic space of \( E. coli \) may be displayed on the surface of filamentous phage, especially when using two-gene phagemid systems (Barbas, 1993) Human growth hormone (Bass et al., 1990), trypsin inhibitor (Roberts et al., 1992), ricin B-chain (Swimmer et al., 1992), and alkaline phosphatase (AP) (McCafferty et al., 1991, Light and Lerner, 1992), have all been expressed It is also possible to construct systems expressing two different proteins, for example, the expression of (alkaline phosphatase) AP-pVIII and Fab-pIII fusions These phage can be used directly as immunological reagents (Light and Lerner, 1992) This technology may
also be used to examine enzyme activities, rapid identification of highly active and selective protease substrates for both stromelysin and matrilysin was possible using a random hexamer library in the fAFF-1 phage (Smith et al., 1995). This method can be used to isolate new substrates for poorly characterised endoproteases.

Human neutrophil elastase (HNE) is an abundant serine protease involved in the elimination of pathogens and in connective tissue restructuring. Hereditary reduction of the circulating α₁ antiproteinase inhibitor, the principal physiological inhibitor of HNE, or inactivation of α₁ antiproteinase inhibitor by oxidation results in extensive destruction of lung tissue, caused by the uncontrolled elastolytic activity of HNE. For this reason it was desirable to identify effective inhibitors of HNE which could be of therapeutic value. A library of pIII fusion phage displaying 1000 customised protease inhibitor variants derived from wild-type bovine pancreatic trypsin inhibitor (BPTI), was created. One inhibitor was isolated which was 50-fold more potent than the most potent anti-HNE inhibitor (Roberts et al., 1992).

1.8.3.4 Constrained Libraries

Most small peptides that bind specifically to target proteins in vivo are constrained, usually by disulphide bridges. 'Constrained' peptide libraries have been constructed, in which the potential conformation of the displayed peptide is limited by the structural constraints built around it, for example, flanking the random sequence by two cysteine residues forces the displayed peptide into a loop conformation. This type of library may be required to overcome the problem of mimicking discontinuous epitopes with linear, unconstrained peptides (Cortese et al., 1994). Constraining the random sequence still permits flexibility within the loop, however, constraining into an incorrect conformation could also prevent binding of certain selector molecules.

Constrained libraries could prove to be richer sources of mimotopes than linear peptides. In one particular study an antibody with a linear epitope selected it's recognition sequence from a linear hexapaptide library but also selected unrelated clones with cysteines at the first and sixth positions. This suggests that these were mimotopes constrained by a disulphide bond (Matthews and Wells, 1993). The residues of a disulphide-close loop (DCL) are only partially constrained by the disulphide bond, the
larger the loop the less the constraint. The amino acid sequence within the loop can also impose additional constraint (Cunningham et al., 1994). Sequences rich in Pro, Val or Ile are more constrained than are sequences rich in Gly, Ala or Ser. The formation of helices or the burial of hydrophobic residues can also limit the freedom of the main chain. Linear peptides that mimic the binding of a conformational epitope, or protein-protein binding have been found but are rare (Ladner, 1995). A constrained peptide (CP) can show higher affinity for a target than an unstructured peptide (UP), a ligand that has the shape to complement a binding site should have higher affinity than a similar ligand that changes shape when it is separated from the target. The more tightly constrained a peptide segment is the less likely it is to bind any particular target, however, if it does bind, it is more likely to be tighter and more specific. It is desirable to have varied peptides that are constrained in several different frameworks of varying rigidity. It has also been found that CPs display far more diversity than UPs, and it is thought that having 6 optimal amino acids in a CP, that are correctly positioned can lead to specific binding to macromolecular targets (Parmley and Smith, 1988).

The first example of the use of phage-displayed peptide libraries to identify a structural epitope was by Hoess et al., 1994, previous to this little success was had in identifying conformational epitopes. In this study two hexapeptide phage libraries were screened with mAb CB5B10, raised against plasminogen activator inhibitor type-1 (PAI-1). This protein is a member of the serine protease inhibitor (serpin) family and the principal physiologic inhibitor of both tissue-type and urokinase-type plasminogen activators (Ny et al., 1986). One of the libraries was contrained to form loops by disulphide bond formation between flanking cysteine residues. An equal mixture of both libraries was used during the biopanning procedure, the consensus sequence selected matched residues 115-119 of PAI-1. Only phage from the constrained library were selected by the mAb. This suggests that constraining the peptide is required for bonding. This was investigated further by mutating the selected sequence, replacing one of the cysteine residues with a serine, thus eliminating the disulphide bond and the loop formation. The efficiency of capture of the serine-substituted peptide was two orders of magnitude less than that for the original phage containing the disulphide bond. This suggests that the selected peptide not only required the appropriate side chains for binding, but that they be held in a loop.
conformation to mimic the structure found in PAI-1 Constraining the peptide confers the advantage of restricting the peptide to fewer conformations, allowing the determination of more structurally complex epitopes

A study by Felici et al., 1993, found a mimic for a discontinuous epitope, similarly, both a constrained and unconstrained library were used, but not simultaneously. The mAb 1B7 which neutralises *Bordetella pertussis* by binding the enzymatically active subunit S1 of the *Bordetella pertussis* toxin (PTX), was the selecting antibody in this case. A precise identification of the antigen residues involved in the binding of the mAb 1B7 could not be obtained although some similar di- or tri-peptide identities could be found in the comparison with the sequence of the S1 subunit of PTX. The original antigen was able to compete for antibody binding with the selected phage clones, this indicates that binding occurs at the level of the antigen binding site. However, *in vivo* immunisation with phage particles did not prove successful in eliciting a protective immune response, a more complex and sophisticated library may be required to achieve this goal. The methods used for selecting mimotopes with mAbs tend to drive selection toward structures which show the 'best fit' to the ligate, and may not actually correspond to efficient immunogens.

Japanese encephalitis virus (JEV) has also been the focus for identification of conformational epitopes using peptide display technology (Hirabayashi et al., 1996). Synthetic libraries were used with two monoclonal antibodies that recognise conformational epitopes on the prM protein of JEV. Synthetic libraries can contain the 20 genetically encoded natural L-amino acids as well as D-optical isomers and unnatural amino acids, giving greater diversity than phage libraries. Two consensus sequences were identified, but only one was capable of producing antisera, on immunisation, which recognised the virus. Short peptides have poor immunogenicity and are usually conjugated to a carrier protein to prepare anti-peptide antisera, this procedure can modify the antigenic determinants making it difficult to obtain antibodies which could recognise the original protein. Identification of a specific peptide for a neutralising MAb may produce polyclonal antibodies which have virus-neutralising activity.
Phage display has also been used to map the surface of many viral antigens, such as the hepatitis B surface antigen (HBsAg) (Chen et al., 1996). HBsAg is the diagnostic marker for Hepatitis B (HBV), which causes major endemic illness throughout the world and is associated with a greatly increased frequency of primary hepatoma, a major cancer in the world (Hollinger, 1990). HBsAg is the major component of the envelope of the virus and is mainly present in the plasma of HBV-infected patients (Mangold and Streeck, 1993). Four monoclonal antibodies were used in the study, three of which were found to bind to distinct discontinuous epitopes between residues 101 and 207 of HBsAg, the fourth bound residues 121-124. The data obtained was used to map the surface of the antigen and to derive a topological model for the α-carbon trace of the 101-207 region of HBsAg. This approach would be useful for mapping other proteins, where the crystal structure is not available but a set of monoclonal antibodies are accessible. A detailed picture of the amino acids that make up a particular epitope can be obtained for most Ab-Ag interactions via x-ray crystallography (Amit et al., 1986), chemical modification (Jemmerson and Patterson, 1986), or deuterium exchange analysis by nuclear magnetic resonance (NMR) (Patterson et al., 1990). These methods require substantial effort and are very difficult technically, the phage display approach has proven a useful alternative for epitope identification.

1.8.3.5 Identification of Disease-specific Mimotopes using Human Sera

It has been possible to identify mimotopes of pathological antigens using only peptide libraries and human sera (Folgorni et al., 1994). In this way, diseases for which the aetiological agent is unknown, can be investigated using patient sera, thus improving the possibilities for the diagnosis and prognosis of these diseases. Identification of disease-specific mimotopes could provide a set of novel reagents for the diagnosis of the disease. In cases where the humoral immune response is protective, the isolated phagotopes could also represent a step in the development of acellular vaccines. A model system was used to investigate the potential of this methodology, sera from both human hepatitis B virus envelope protein (HBsAg)-immunised individuals (positive serum) and non-immunised individuals (negative serum) were used. This antigen was chosen as it is known that immunisation with HBsAg can elicit a protective immune response.
The experimental strategy consisted of the following steps

- Affinity selection of a peptide library with a positive serum,
- Screening of the enriched phage population for epitopes also recognised by a second positive serum,
- Further screening of the selected phagotopes using large panels of positive and negative sera

Screening the affinity-selected phage with a patient serum different from that used for the enrichment allows only those phage displaying epitopes recognised by antibodies of 'common' specificity between the two sera to be identified. There should be a high percentage of disease-specific phagotopes among the common ones. It was not possible to subtract completely all the phage which reacted with the sera of healthy individuals, this may have been due to a low efficiency of the selection procedure. Phage bearing HBsAg peptide mimics were identified, and used as immunising agents, which proved to be both antigenic and immunogenic mimics of the natural antigen HBsAg. This model system proves the potential of this simple and inexpensive procedure for 'footprinting' the immune response against a particular antigen, even in the absence of any information on the aetiological agent, the only requirement being clinically well-characterised sera from patients and normal individuals.

1.8.4 Advantages of using a Panel of RPLs

It has been seen that the peptides selected by a given selector molecule are significantly influenced by the structural framework within which a random peptide is presented. This has been further investigated by the construction of a panel of peptide libraries comprising a variety of structurally constrained and unconstrained peptides of varying length, and screening it with a number of monoclonal antibodies (MAbs) and polyclonal antibodies (PCAbs) (Bonnycastle et al., 1996). High affinity peptides are more likely to be found by screening a panel of peptide libraries, since a single library will not provide tight-binding peptides for all antibodies, and the preference of an antibody for particular constraints is not usually known in advance. Eleven libraries were screened with 17 antibodies, 15 MAbs against peptides, linear and discontinuous epitopes on proteins or carbohydrates and 2 PCAbs against proteins of known structure. All except 2 MAbs isolated binding peptides from several libraries, the 2 which failed to select peptides were
directed against discontinuous protein epitopes. Only a few of the large fraction of antibodies that bind to discontinuous epitopes in polyclonal serum were able to isolate weakly binding, cross-reactive peptides, while the less abundant antibodies that recognise linear epitopes cross-reacted well with peptides. Many of the antibodies showed polyspecificity in their preference for different consensus sequences within and between libraries. No library produced the best-binding phage across all of the antibodies, but the best-binding phage pools were obtained from a few of the libraries, especially if the binding was weak or moderate across the panel. These results were determined using the average binding strength of each library as a measure. These values were calculated by adding the scores within a library from every screening and dividing by the number of screenings performed on the library. All of the libraries had approximately the same average binding strength.

This study and previous work have also shown that the majority of anti-protein antibodies that cross-react with peptides recognise linear epitopes on their corresponding immunogens, and these epitopes usually occur on the folded protein as turns and extended loops (Getzoff et al., 1987; Geysen et al., 1987; Tainer et al., 1984). Discontinuous epitopes on folded proteins usually bind antibodies over a large shallow surface, this may be a reason for their inability to select peptides from a phage library. Large shallow binding sites would not allow a peptide to become buried as occurs with linear epitopes (Wilson et al., 1994). Discontinuous epitopes may be more successfully mimicked by folded proteins than by peptides. A more complex library displaying large inserts that are part of a protein having a very stable fold, is likely to be an important development. In this way an epitope or mimotope which would better fit the large, shallow binding site of a discontinuous epitope may be selected (Bonnycastle et al., 1996).

1.8.5 Gene-Fragment Libraries

A recent study was carried out to compare the efficiencies of epitope mapping by gene-fragment libraries and two random peptide libraries in combination with peptide scan (Fack et al., 1997). The gene-fragment libraries were constructed by cloning DNasel digested random gene fragments into the 5' terminus of the pIII gene of fd phage. The random peptide phage libraries displayed 6 mer and 15 mer peptides at the N-terminus of
the pIII protein. Peptide scanning required the synthesis of oligopeptides on activated membranes using the SPOTs system (Genosys, Cambridge, England). Four monoclonal antibodies were used, all were thought to recognize linear epitopes, shown by immunoblot, however, a positive reaction on immunoblots can also be achieved by non-linear epitopes where the reacting portions are in relatively close proximity. Using the gene-fragment display system a single round of affinity selection resulted in 65% to 95% ELISA-positive clones and in the determination of epitopes recognized by all 4 of the MAbs. However, after 2-4 rounds of biopanning with the 6 mer library, only 2 of the 4 antibodies yielded positive clones and 3 out of 4 antibodies failed with the 15 mer library. The epitopes were fine mapped by peptide scan, this showed that the minimal epitopes of the two antibodies which selected clones from the 6 mer library, MAbGDO5 and MAbp53-11, consisted of four and five amino acids respectively. The antibodies MAb215 and MAbL13F3 failed to yield the epitope sequence from either random peptide library. For the determination of the MAb215 epitope by gene-fragment phage display a minimal overlap size of 17 amino acids was obtained and the peptide scan narrowed this region to 11 amino acids. This explains why the MAb215 epitope could not be found in the 6 mer library. Neither the random peptide library or the peptide scan succeeded in yielding an epitope sequence for MAbL13F3. The sequence recovered from the gene-fragment library was relatively large and it is possible that this antibody recognizes an epitope larger than the 15 amino acids used in the peptide scan or that the antibody recognizes structures with a high conformational constraint. It was found that in general when an antibody produced a positive signal in an immunoblot it also yielded positive clones by gene-fragment phage display. Antibodies that could not detect their antigen by immunoblot, also failed to select positive clones from the gene-fragment libraries, these antibodies recognized conformational epitopes.

Random peptide libraries cannot contain all possible epitopes and therefore will not yield positive clones for all antibodies investigated. However, short epitopes can be identified in most cases using random peptide libraries. It appears that epitopes spanning more than six consecutive amino acids can be more reliably detected by gene-fragment libraries, and epitopes with particular structural conformations which often escape detection by random peptide libraries or peptide scan can be determined.
1.9 Aims of this Thesis

This thesis may generally be divided into two parts. Both possess a common theme in that each uses recombinant DNA methods to address important aspects related to the diagnosis and immune-response to EBV-related disease.

In the first part, a number of EBV antigens were cloned into a plasmid for expression in *Escherichia coli*. Using the chosen system, which is described in section 1.7.1, antigens are expressed in a soluble form with the *E. coli* thioredoxin. To date these antigens have been purified from lysates of mammalian cells in cell culture. Expression of these antigens in *E. coli* will allow production of greater quantities of recombinant antigen at a lower cost, for the purposes of developing a serology-based method for diagnosing EBV-related diseases. Serotyping allows the determination of the type of EBV infection an individual has at a particular time, i.e. primary, past or reactivated infection.

In the second part we have attempted to use phage display technology to identify peptides which bind to the EBV neutralising antibody 72A1. To this end, a number of random phage libraries were screened with the virus-neutralising anti-gp350 monoclonal antibody, 72A1, using different ‘biopanning’ methods.

Successful identification of the epitope or mimotopes of this antibody would have potential applications in the area of vaccine development. Synthetic peptides with high affinity for 72A1 could be produced and used as constituents of a vaccine against EBV-related disease.
Chapter 2

Materials and Methods
2.1 Biological materials

2.1.1 Antibodies

72A1, F29.167, and F2.1 were supplied by Dr. Andrew Morgan, University of Bristol. These antibodies were provided as monoclonal cell line supernatants which were purified on a Protein G column then dialysed in PBS, but not exhaustively. They were stored at -20°C. Polyclonal anti-gp350/220, MDP 61.1.4.

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse-alkaline phosphatase (AP) conjugate</td>
<td>Promega</td>
</tr>
<tr>
<td>Anti-mouse-horseradish peroxidase (POD) conj.</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Anti-fd bacteriophage</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-human-AP conjugate</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Anti-rabbit-AP conjugate</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Anti-human IgG-POD (λ-chain specific)</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-human IgM-POD (μ-chain specific)</td>
<td>Dako</td>
</tr>
<tr>
<td>GullSORB</td>
<td>Gull Laboratories</td>
</tr>
</tbody>
</table>

2.1.2 Serum Samples

All anti-EBV IgM-positive sera were supplied by Mrs. Dorothy Wyatt, Regional Virus Laboratory, the Royal Hospitals Trust, Belfast. All these sera were shown to be strongly positive for anti-EBV IgM by indirect immunofluorescence (Gull Laboratories), after removal of rheumatoid factor. They were not tested for blood-borne or other viruses.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Patient details</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age</td>
</tr>
<tr>
<td>962/97*</td>
<td>35yrs</td>
</tr>
<tr>
<td>1385/97*</td>
<td>35yrs</td>
</tr>
<tr>
<td>7126/97</td>
<td>18yrs</td>
</tr>
<tr>
<td>7217/97</td>
<td>53yrs</td>
</tr>
<tr>
<td>9217/97</td>
<td>26yrs</td>
</tr>
<tr>
<td>10211/97</td>
<td>26yrs</td>
</tr>
<tr>
<td>12874/97</td>
<td>18yrs</td>
</tr>
</tbody>
</table>
All EBNA IgG-positive and IgG-negative sera, and EB VCA IgM-positive and IgM-negative sera, were supplied by Carol Mongan, Virus reference laboratory, UCD, Belfield. All specimens were from 1997.

<table>
<thead>
<tr>
<th>EBNA IgG-negative</th>
<th>EBNA IgG-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>6627 6737</td>
<td>7248 7256 7467</td>
</tr>
<tr>
<td>6769 6891</td>
<td>7302 7310 8090</td>
</tr>
<tr>
<td>6985 6988</td>
<td>7343 7344 8712</td>
</tr>
<tr>
<td>7068 7127</td>
<td>7387 7456</td>
</tr>
<tr>
<td>8725 8694</td>
<td>7529 7971</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EB VCA IgM-negative</th>
<th>EB VCA-IgM positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>7523 7266</td>
<td>7611 7617</td>
</tr>
<tr>
<td>7268 7271</td>
<td>7739 7837</td>
</tr>
<tr>
<td>7274 7281</td>
<td>8128 8318</td>
</tr>
<tr>
<td>7291 7296</td>
<td>8413 8415</td>
</tr>
<tr>
<td>7299 7300</td>
<td>8811</td>
</tr>
</tbody>
</table>

All normal serum were supplied by a Dublin Hospital, July 1998 (samples were taken for routine clinical investigations)

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7435 7436</td>
<td>7458 7464</td>
<td>7438 7439</td>
<td>7459 7465</td>
</tr>
<tr>
<td></td>
<td>7442 7443</td>
<td>7460 7466</td>
<td>7447 7448</td>
<td>7461 7467</td>
</tr>
<tr>
<td></td>
<td>7449 7454</td>
<td>7462</td>
<td>7456 7457</td>
<td>7463</td>
</tr>
</tbody>
</table>

CMV (Cytomegalovirus) positive sera (BIOTRIN, Dublin)

<table>
<thead>
<tr>
<th>NY1</th>
<th>NY2</th>
<th>NY3</th>
<th>NY4</th>
<th>NY5</th>
</tr>
</thead>
<tbody>
<tr>
<td>8638</td>
<td>16792</td>
<td>69/95</td>
<td>0124</td>
<td>0623</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>769/95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>607/95</td>
<td></td>
<td>765/95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10190</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
EBV positive sera (BIOTRIN, Dublin)

MS8    MS9
2664    CAC6385
2656    1318/95
26637   331/95

2.1.3 Oligonucleotides

R&D Systems and Genosys

**EBNA1**

Forward primer

5' CGG GAT CCC AGG AGT CCC AGT AGT CAG TCA 3'

Reverse primer

5' CGG GAT CCT AAT ACG ATT GAG GGC GTC TCC TCC 3'

**p18VCA**

Forward primer

5' GTT ATG GAT CCC CGG CTG CCC AAG 3'

Reverse primer

5' GTT TCG GAT CCT CTA CTG TTT CTT 3'

**BARF0**

Forward primer

5' TGC CAG TGG GAT CCC GGG TAC GCT 3'

Reverse primer

5' TAC GGG GAT CCT AAA GTC GAT GTA 3'

**Early antigen**

Forward primer

5' CGG GAT CCC GAA ACC ACT CAG ACT CTC 3'

Reverse primer

5' CGG GAT CCC GAG AAC ATG GTG TTA AAT 3'

2.1.4 Commercial Kits and Enzymes

Thiofusion cloning kit        Invitrogen
QIAEX         QIagen
Phage libraries      New England Biolabs (NEB)
T7 Sequencing kit Pharmacia
Protein assay dye reagent BIORAD
Supersignal substrate Pierce

All restriction and deglycosylation enzymes were supplied by Boehringer Mannheim

2.2 Chemical materials

Protein prestained markers NEB
S$^3$ labeled dATP Amersham
dNTPs Pharmacia Biotech
Marvel Premier Beverages
Enterokinase Bioyme labs ltd
Chloroform ROMIL
Dimethyl formamide Riedel-de-Haen

Sigma Chemical Co,
NP-40, Urea, Dithiothreitol, Coomassie blue R, BCIP/NBT, Streptavidin, Kanamycin,
Gelatin, Tetracycline, NZ amine A, Ethanolamine, Biotin-NHS, Liquid Phenol,
Tryptophan, Nitocellulose, Cassamino acids, Ampicillin, Potassium acetate, Tween-20,
Thiamine, Sodium bicarbonate, BSA, dialysed BSA, Sodium azide, PEG-8000, Sodium
iodide, Sigma fast pNPP, Sigma fast OPD, Ammonium sulphate, Sigmacote, Ammonium
phosphate, Mineral oil, PMSF

Merck,
Boric acid, Ammonium persulphate, Sodium acetate, Magnesium chloride, Glucose,
Sodium chloride, Potassium chloride, Sodium hydroxide, Sodium dodecylsulphate,
Calcium chloride, Glycine, Ammonium chloride

BDH,
TEMED, Bromophenol blue, Potassium dihydrogen phosphate, Potassium hydrogen
phosphate, Sodium phosphate, Glycerol, Tris(hydroxymethyl)methylamine, EDTA,
Magnesium sulphate, Mercaptoethanol, Ethidium bromide, Sucrose, Isoamyl alcohol,
Hydrochloric acid, Acetic acid, Methanol, Isopropanol
Boehringer Mannheim;
Protein-G agarose, Agarose, Low melt agarose, IPTG, Leupeptin.

Oxoid;
Agar technical, Tryptone, Yeast extract.

KODAK;

National diagnostics;
Acrylagel, Bis-acrylagel.

Gibco-BRL;
1Kb DNA ladder, β-galactosidase (X-gal).

2.3 DNA Manipulation
Solution and reagents as detailed in appendix section I(i).
All centrifugation of microfuge tubes was carried out in a Biofuge 13 from Heraeus Instruments, all centrifugation of 50 ml Falcon tubes was carried out in a Labofuge from Heraeus Instruments.

2.3.1 Storage of DNA Samples
DNA samples should ideally be stored in TE buffer pH 8.0 at 4°C. The use of EDTA chelates heavy metal ions that are needed for DNase activity while storage at pH 8.0 minimises deamidation.

2.3.2 Equilibration of Phenol
Before use, phenol was equilibrated to pH 8.0 as DNA partitions into the organic phase at <pH 7.8
1. Solid phenol was melted at 68°C, and hydroxyquinoline was added to a final concentration of 0.1% (acts as an antioxidant, a chelator of metal ions, and an RNase inhibitor).
2 An equal volume of buffer (0.5 M Tris Cl pH 8) was added to the liquefied phenol and stirred for 15 minutes. The two phases were then allowed to equilibrate and as much as possible of the upper aqueous phase was removed.

3 The extraction was repeated using equal volumes of 0.1 M Tris Cl pH 8 until the pH of the phenol was > 7.8.

4 An equal volume of 0.1 M Tris Cl pH 8 and 0.2% β-mercaptoethanol were added to the phenol, which was then stored at -20°C in the dark.

2.3.3 Phenol/Chloroform Extraction

1 An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added to the DNA solution, mixed by vortexing and centrifuged for 5 min at 13000 rpm.

2 The upper aqueous phase was removed, taking care not to take any material from the interphase, this was placed in a fresh microfuge tube.

3 To this an equal volume of chloroform/isoamylalcohol (24:1) was added, vortexed as before and centrifuged for 5 min at 13000 rpm. Again the upper aqueous phase was removed to a fresh tube.

2.3.4 Sodium acetate/Ethanol Precipitation of DNA

Concentration of nucleic acid samples or changing the buffers in which a sample is dissolved was achieved by ethanol precipitation.

1 One tenth volume of 3 M sodium acetate (pH 5.2) was added to the solution of DNA, vortexed and 2 volumes of 100% ethanol were added. Again this was vortexed, and the mix was incubated at -20°C overnight or at -80°C for 30 min.

2 The DNA preps were then centrifuged for 30 min at 13000 rpm at 4°C, the supernatant was removed and the pellet was washed with 1 ml 70% ethanol to remove excess salts.

3 The tube was centrifuged for 5 min at 13000 rpm, the supernatant was removed and the pellet was allowed to dry for approximately 30 min.

4 The pellet was resuspended in an appropriate volume of sterile Tris-EDTA (pH 8.0) (TE)
2.3.5 Ammonium sulphate/Isopropanol Precipitation of DNA

This method was used for PCR products which were cut from agarose gels and purified by the QIAEX II kit.

1. To the PCR reaction mix a ¼ volume of 10 M ammonium acetate was added, the solution was vortexed and an equal volume of 100% isopropanol was added. The solution was vortexed and incubated at -20°C for at least 1 hr or overnight.

2. Tubes were centrifuged for 30 min at 13000 rpm at 4°C, the supernatant was removed and the pellet was washed with 1 ml 70% ethanol, and centrifuged for 5 min at 13000 rpm.

3. The supernatant was removed, the pellet was allowed to dry for 30 min, and was then resuspended in an appropriate volume of sterile TE (pH 8.0).

Notes

(a) To remove SDS from a sample, the sample can be adjusted to 0.2 M sodium chloride before the addition of ethanol.
(b) If the DNA to be precipitated is small (≤200 bp), the sample should also be made 10 mM in magnesium chloride before ethanol precipitation.

2.3.6 Spectrophotometric Quantification of DNA

In general, for quantification of maxiprep DNA 5 μl of DNA was added to 495 μl of distilled water. Absorbance readings were taken at 260 nm and 280 nm. An absorbance reading at 260 nm of 1 corresponds to approximately 50 μg/ml of double-stranded DNA. The ratio between the readings at 260 nm and 280 nm provides a measure of the purity of the DNA. A pure preparation of DNA will have a 260/280 ratio of between 1.8 and 2.0.

2.3.7 Agarose Gel Electrophoresis of DNA

Electrophoresis through agarose gels is the standard method used to separate, identify, and purify DNA fragments. The technique is simple, rapid to perform, and can be used for the isolation of DNA fragments. Typically, the following procedure was used:

1. An appropriate quantity of agarose or low melt agarose was added to 100 ml 1X TBE buffer. The amount of agarose depends on the percentage agarose required. This was decided in relation to the size of the DNA fragments being separated.
2 The mixture was boiled to dissolve the agarose, when cooled sufficiently (~60°C) the gel was poured into the mold and the comb inserted

3 The gel was allowed to set before filling the chamber with 1X TBE, the comb was then removed

4 To a 20 µl sample, 4 µl of sample buffer was added and loaded into the wells made by the comb. Sample buffer was also added at 1X concentration to 300 ng 1 Kb DNA ladder which was loaded into the first well.

5 The gel was run at constant voltage (5 V/cm) for 1-2 hr. When complete the gel was stained in ethidium bromide (0.5 mg/ml) for 30 min, placed in water to destain for 15 min and viewed under UV illumination.

2.3.8 Isolation of DNA from Agarose Gels

1 Low melting point agarose gels were prepared in 1X TAE buffer (gel isolation is not carried out in TBE buffer as borate ions are difficult to remove from the resultant DNA solution).

2 After electrophoresis, the gels were stained in an ethidium bromide bath and viewed under 70% UV illumination. The time of exposure to UV light was kept to a minimum, as overexposure to UV causes damage to the DNA.

3 The DNA band to be isolated was excised from the gel using a scalpel and placed in a sterile microfuge tube. Excess agarose was cut away to minimise the size of the gel slice.

2.3.9 QIAEX II Agarose Gel Extraction Protocol (QIAGEN)

1 A 1.5 ml microfuge tube was used for up to 250 mg of agarose. The weight of the gel slice was determined and 3 volumes of buffer QX1 was added to 1 volume of gel.

2 The QIAEX II was resuspended by vortexing for 30 s, and then 10 µl was added to the microfuge tube with the gel. This was incubated for 10 min at 50°C to solubilise the agarose and bind the DNA. The tube was vortexed every 2 min to keep the QIAEX II in suspension.

3 The sample was then centrifuged for 30 s and the supernatant was removed. The pellet was washed once with 500 µl of QX1 and twice with 500 µl of buffer PE. The pellet was air-dried for 15 min until white.
4 Twenty microlitres of sterile distilled water was added to resuspend the pellet, and the tube was vortexed, followed by incubation at room temperature for 5 min.

5 The sample was centrifuged for 30 s and the supernatant was carefully removed to a fresh tube.

6 A further 20 µl of sterile distilled water was added and the pellet was resuspended and vortexed. The tube was centrifuged and the supernatant removed to the fresh tube.

2.3.10 Decontamination of Ethidium Bromide Solutions

This method was developed by Lunn and Sansone (1987).

1 Sufficient water was added to reduce the concentration of ethidium bromide to <0.5 mg/ml.

2 A 0.2 volume of fresh 5% hypophosphorus acid and 0.12 volume of fresh 0.5 M sodium nitrate was then added. The solution was mixed carefully and the pH was checked (should be <3).

3 After incubation for 24 hr, a large excess of sodium bicarbonate was added and the solution was discarded.

2.3.11 Restriction Digestion of DNA

Restriction enzymes bind specifically to and cleave double stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition site. The restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X).

1 DNA digests were performed by adding 1 unit of enzyme/µg of DNA solution.

2 The reaction mix was vortexed briefly and centrifuged, then incubated for 2 hours at the optimum enzyme temperature (usually between 30°C and 37°C).

3 The final DNA concentration in the digest should be between 100 and 300 ng/ml for optimal digestion.

2.3.12 Dephosphorylation of Linearised Plasmid DNA

1 To the digested DNA (5 µg in 20 µl) 1 µl of calf intestine alkaline phosphatase (CIP) was added, 15 µl of the 10X enzyme buffer was added and the volume was made up.
to 150 µl with sterile distilled water (CIP was added 1 unit/100 pmoles for cohesive termini and 1 unit/2 pmoles for blunt termini)

2 The solution was vortexed, centrifuged briefly and incubated for 30 min at 37°C. This was followed by denaturation by heating to 75°C for 10 min.

3 This DNA was cleaned by phenol/chloroform extraction and ethanol precipitation.

2.3.13 Ligation of DNA Molecules

Several strategies for ligation of DNA molecules were used depending on the nature of the termini.

(1) Cohesive termini

Ligations of equimolar amounts of vector and insert DNA (<1 µg) were carried out overnight at 16°C in a commercial ligation buffer (5 mM ATP) with 10 Weiss units of ligase/ml. After ligation, the samples were heated to 10 min at 70°C to inactivate the ligase (this appears to improve transformation efficiencies).

During ligation, T4 DNA ligase will catalyse the formation of a phosphodiester bond between adjacent nucleotides only if one contains a 5' phosphate group and the other contains a 3' hydroxyl group. Recircularisation of plasmid DNA can therefore be minimised by removing the 5' phosphate groups by treatment with calf intestinal phosphatase enzyme (CIP).

Typical ligation reaction

<table>
<thead>
<tr>
<th>per 10µl reaction</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X enzyme buffer</td>
<td>1</td>
</tr>
<tr>
<td>vector DNA (&lt;1 µg)</td>
<td>2</td>
</tr>
<tr>
<td>PCR DNA (&lt;1 µg)</td>
<td>2</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1</td>
</tr>
<tr>
<td>sterile distilled water</td>
<td>4</td>
</tr>
</tbody>
</table>
Small scale Preparation of Plasmid DNA (Miniprep)

Solution and reagents as detailed in appendix section I(n).

This is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981)

Harvesting

1. A single bacterial colony was used to inoculate 5 ml of medium (with appropriate antibiotic), and incubated overnight at 30°C/37°C

2. 1.5 ml of this culture was poured into a sterile microfuge tube and centrifuged for 30 s at room temperature, the remainder was stored at 4°C. The medium was removed from the tube, leaving the pellet as dry as possible.

Lysis

1. The pellet was resuspended thoroughly in 100 μl of solution I by vigorous vortexing. To this 200 μl of freshly prepared solution II was added, the tube contents were mixed by inverting the tube rapidly a number of times. 150 μl of ice-cold solution III was added and vortexed gently for 10 s.

2. The lysate was centrifuged for 5 min at 13000 rpm, and the supernatant was transferred to a fresh tube, taking care not to carry over any of the white precipitate.

3. An equal volume of phenol/chloroform/isoamyl alcohol (25 24 1) was added, mixed by vortexing and centrifuged for 5 min at 13000 rpm.

4. The upper aqueous phase was removed to a fresh tube, to which 2 volumes of 100% ethanol was added, the solution was vortexed and centrifuged for 5 min at 13000 rpm.

5. The supernatant was discarded, the pellet was washed with 1 ml 70% ethanol, centrifuged as before and the supernatant was removed again.

6. The pellet was allowed to air-dry, and was then resuspended in 50 μl of TE (pH 8.0), 1 μl of DNase-free RNase A (20 μg/ml) was also added, vortexed and stored at 4°C.
2.3.15 Large scale Preparation of Plasmid DNA (Maxiprep)

Solution and reagents as detailed in appendix section I(ii).

1. A 400 ml bacterial culture was grown overnight at 37°C with vigorous agitation. The culture was centrifuged for 10 min at 7974 g in a Sorvall/Beckman centrifuge at 4°C.

2. The bacterial cell pellet was resuspended in 20 ml solution I. This was chilled on wet ice and 40 ml of solution II (fresh) was added, mixed gently but thoroughly and incubated on wet ice for 5 min. Twenty millilitres of solution III was added, mixed gently but thoroughly until all viscosity was gone, and incubated on ice for 5 min.

3. This suspension was centrifuged for 15 min at 13180 g at 4°C, meanwhile 40 ml of isopropanol was added to a clean centrifuge bucket. The supernatant was added to the isopropanol, without carrying forward any white precipitated material. This was done by filtering through muslin gauze, followed by incubation at room temperature for 15 min (At this point the pellet could be stored at -20°C overnight).

4. The mixture was centrifuged at 8000 rpm for 30 min at 4°C, the supernatant was removed and the bucket was inverted on some paper to drain.

5. The pellet was resuspended gently in 4 ml TE pH 8.0, RNase A was added to a final concentration of 25 μg/ml, and incubated at 37°C for 1 hr.

6. This was then extracted twice with an equal volume of phenol/ chloroform/isoamylalcohol (25:24:1) and once with chloroform/isoamylalcohol (24:1), taking care not to take any interphase material.

7. For each extraction the solution was centrifuged for 5 min at 1912 g at 4°C. 0.4 ml of 3 M sodium acetate was added, mixed and added 9 ml of 100% ethanol.

8. This was vortexed and incubated at room temperature for 10 min, the DNA was pelleted by centrifugation for 30 min at 13000 rpm at 4°C.

9. The pellet was washed in 70% ethanol, centrifuged for 5 min at 13000 rpm at 4°C, and resuspended in 1 ml TE pH 8.0.

2.3.16 Polymerase Chain Reaction Protocol (PCR)

The polymerase chain reaction (PCR) involves the amplification of specific DNA sequences using DNA primers which anneal to the DNA of interest. The primers are designed so that one anneals to the forward DNA strand and the other anneals to the reverse strand thus allowing polymerisation of both strands by the enzyme Taq DNA...
polymerase. This results in exponential amplification of the sequence of interest. The standard requirements for a PCR reaction mix are detailed below:

<table>
<thead>
<tr>
<th>Per 50 μl reaction</th>
<th>μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile ultra pure water</td>
<td>37</td>
</tr>
<tr>
<td>10X enzyme buffer</td>
<td>5</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP 10 mM mix</td>
<td>1</td>
</tr>
<tr>
<td>Forward primer 250 ng/μl</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer 250 ng/μl</td>
<td>1</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1</td>
</tr>
</tbody>
</table>

This reaction mix was overlayed with 50 μl sterile mineral oil before placing in the minicyclicer.

Polymerase Chain Reaction (PCR), standard program, only Ta was varied for each antigen, depending on the primers. Ta is the annealing temperature for the specific primers.

<table>
<thead>
<tr>
<th>Stage no.</th>
<th>Step no.</th>
<th>Temp. (°C)</th>
<th>Time (min)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>94</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>94</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Ta</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>72</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>72</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>15</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

90
2.4 Thiofusion Expression System (Invitrogen)

Solution and reagents as detailed in appendix section II.

2.4.1 Bacterial Strains

*E. coli* G1724 and G1698 have the following genotype F',λ',lacI',lacPL8,ampC::PtrpC'. G1724 was used for expression at 30°C and above while G1698 was used for expression below 30°C. Both strains were maintained on LB agar plates before transformation, after transformation with cloned plasmid DNA both strains were grown on RMG<sub>amp</sub> plates or RM<sub>amp</sub> liquid media.

2.4.2 Preparation of Chemically Competent Cells using Calcium chloride

1. G1724/G1698 was streaked on a fresh LB plate and incubated overnight at 37°C/30°C.

2. A single colony was used to inoculate 5 ml SOB, which was incubated overnight with vigorous shaking at 37°C.

3. To 20 ml SOB, 500 µl of the overnight culture was added followed by incubation with vigorous shaking at 37°C until the OD<sub>500nm</sub> reached 0.37-0.5. (~2.5 hr). The culture was poured into a 50 ml Falcon tube and centrifuged at 2500 rpm at 4°C for 10 min.

4. The supernatant was removed, the cells were resuspended in 10 ml of cold 100 mM CaCl<sub>2</sub> by repeated inversion, and left on ice for 20 min. This solution was centrifuged for 10 min at 2500 rpm at 4°C, the supernatant was again removed and the cell pellet was resuspended in 1 ml cold 100 mM CaCl<sub>2</sub> by gently pipetting up and down.

5. These cells could then be stored for ~5 days at 4°C.

2.4.3 Transformation of Competent Cells

1. A heating block was equilibrated to 42°C. Two hundred microlitre volumes of the competent cells were dispensed into microfuge tubes and kept on ice.

2. A 3-5 µl volume of each ligation reaction was added to a separate tube of competent cells and mixed gently with the pipette tip. For control reactions, 10 ng of each supercoiled plasmid (pTrx and/or pAL-781) was added to a separate tube of cells.

3. Each tube was incubated on ice for 30 min. The cells were heat shocked for 90 s at 42°C in a heat block, then placed on ice for 1-2 min.
4 To each tube 800 µl of room temperature SOC medium was added and incubated with agitation at 30°C for 60 min

5 A 200 µl volume of each transformation mix was plated on RMG-amp plates and incubated at 30°C overnight (may need 2 nights)

2.4.4 Expression and Analysis of Transformants

Ampicillin resistant transformants were picked from the plate and used to inoculate 5 ml RM medium with 100 µg/ml ampicillin. These cultures were incubated overnight at 30°C with vigorous shaking. Plasmid DNA was isolated by miniprep (as described in section 2.3.14) for restriction analysis.

2.4.5 Induction of Positive Clones

1 The clone of interest was streaked on an RMG-amp plate and grown at 30°C overnight.
2 A single colony from the plate was taken to inoculate 5 ml of RM medium with 100 µg/ml ampicillin and incubated at 30°C with shaking overnight.
3 Ten millilitres of fresh induction medium was inoculated with 500 µl of the overnight culture in a 100ml culture flask and grown at 30°C to an OD550nm of 0.5 (~2.5hr).
4 A 1 ml sample was transferred to a microcentrifuge tube and centrifuged for 5 min at maximum speed. This was the time zero sample before induction. The supernatant was removed and the cell pellets were stored at -20°C until ready for analysis.
5 To the remainder of the culture tryptophan was added to a final concentration of 100 µg/ml using the 10 mg/ml stock solution (10 µl of stock per 1 ml of culture).
6 The culture was then incubated at 37°C with shaking for 4 hr. The OD550nm was measured after 4 hr and 1 ml samples were taken and treated as for the time zero samples.
7 At this point the crude samples were then analysed by SDS-PAGE or further purification was carried out.
8 The cell pellets for each clone were resuspended in 500 µl osmotic shock solution 2 and kept on ice. A hand held sonicator with a micro-tip was used to sonicate each sample one at a time with three 10sec bursts.
The lysates were put in liquid nitrogen after sonication to flash freeze them, this was followed by quick thawing at 37°C. Two further rapid sonication-freeze-thaw cycles were carried out.

After the last thaw, all samples were centrifuged at 13000 rpm for 5-10 min at 4°C to pellet cell debris and insoluble matter. The supernatants were transferred into fresh tubes and the pellets from the previous step were resuspended in 500 μl of osmotic shock solution 2.

All samples were stored on ice until analysed by SDS-PAGE.

2.4.6 SDS Polyacrylamide Gel Electrophoresis (PAGE)

Solution and reagents as detailed in appendix section III

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>10% Resolving Gel (ml)</th>
<th>12% (ml)</th>
<th>7.5% (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrylamide</td>
<td>3.33</td>
<td>4.00</td>
<td>2.50</td>
</tr>
<tr>
<td>bis-acrylamide</td>
<td>1.35</td>
<td>1.62</td>
<td>1.00</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>ultrapure water</td>
<td>2.62</td>
<td>1.68</td>
<td>3.80</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>5% Stacking Gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrylamide</td>
<td>0.42</td>
</tr>
<tr>
<td>bis-acrylamide</td>
<td>0.168</td>
</tr>
<tr>
<td>1 M Tris (pH 6.8)</td>
<td>0.312</td>
</tr>
<tr>
<td>ultrapure water</td>
<td>1.55</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.025</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.025</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

Preparation of PAGE apparatus

Both plates were washed with detergent, rinsed with tap water and with distilled water. They are then wiped in one direction with tissue soaked with 100% ethanol.
The gasket was placed about the ridged plate, the plates were put together and secured with clamps. Stand in an upright position the resolving gel was poured to within 1" of the top of the larger plate and overlayed with 100% ethanol.

When set, the ethanol was removed and the stacking gel was poured. The comb was inserted and allowed to set. 1X running buffer was poured into the gel box, the clamps and gasket were removed and the plates placed in the chamber with the cut plate facing inward, making sure to exclude any air bubbles.

The chamber was filled with running buffer, the samples were loaded and the electrodes were attached. One gel was run at 30 mAmmps for approximately 1 hr.

When complete the plates were removed, separated and the gel was placed in coomassie stain for 30 min, agitating constantly. Then the gel was placed in destain, with constant agitation, until all background staining was removed. The destain was changed as it became saturated with stain.

2.4.7 Purification by Osmotic Shock

The culture was grown, induced and harvested as described in section 2.4.5, (cells were not lysed). The OD @ 550 nm was recorded before harvesting the cells, 4 hr post induction. Fresh cells were used for osmotic shock.

The cell pellet was resuspended in osmotic shock solution #1 to an OD$_{550}$ nm of 5.0. (The OD$_{550}$ nm value recorded before harvesting was used to determine the volume required, using the following formula)

\[ V_r = (\text{OD}_{550 \text{nm of sample}}/5.0) \times V_s, \]

Where:
- $V_r$ = volume to resuspend the cell pellet
- $V_s$ = original sample volume

The cells were incubated on ice for 10 min, followed by centrifugation for 1 min at 4°C, the buffer was then decanted.

The cell pellet was then resuspended in osmotic shock solution #2, using the same volume as in step 2, incubated on ice for 10 min and centrifuged for 10 min @ 4°C.

The supernatant was transferred to a clean tube, and the pellet was resuspended in the same volume of osmotic shock solution #2.

These samples could then be frozen at -20°C or run on SDS-PAGE.
2.4.8 Purification by Heat Treatment

1. The culture was grown, induced and harvested as described in section 2.4.5, cells were also lysed. A heating block was equilibrated to 80°C.

2. Fifty microlitres of the lysate was reserved and kept on ice, this is the zero time point which is not heat treated. Aliquots of 50 µl were placed in microfuge tubes and incubated at 80°C for different lengths of time, from 30 s to 10 min.

3. The heat treated samples were centrifuged at 3000*g for 15 min @ 4°C, to remove precipitated proteins. The supernatants were transferred to fresh tubes and stored on ice.

4. The protein precipitates were solubilised in 50 µl 1X sample buffer, 10 µl aliquots of the supernatant and solubilised precipitates were analysed by SDS-PAGE.

2.4.9 Ammonium sulphate Precipitation of Recombinant Antigens

1. Saturated ammonium sulphate was prepared by adding 400 g ammonium sulphate (AS) to 500 ml distilled water, the pH was adjusted to 6.0. This solution was stirred for 2 hr at 37°C, until the salt was completely dissolved. This solution crystallises at room temperature and it was necessary to heat and stir before each use.

2. A small scale precipitation was carried out first with 500 µl aliquots of partially purified recombinant protein. The saturated AS was added dropwise to the percentage required for a 1 ml solution, and the volume was made up to 1 ml with PBS (a range of percentages were tried).

3. The samples were then stored on ice for 15-60 min, gently mixed at 10 min intervals and centrifuged at 13000 rpm for 10 min. The supernatants were removed to a fresh tube and the pellets were resuspended in 1 ml PBS.

4. Dialysis tubing was boiled for 10 mins in 1 mM EDTA, rinsed thoroughly with distilled water, and stored at 4°C.

5. The samples were dialysed in ~3 L sterile PBS at 4°C overnight, with stirring.

6. The dialysis tubing was cut in ~10 cm pieces; a clip was put at one end the sample was pipetted into the tubing, all air bubbles were excluded and another clip was used to seal the tubing. This was then submerged in the container of PBS and attached to the side of the container with a piece of thread.
The fluid was removed on the following day to a fresh tube for each sample and a fraction of the sample was analysed by SDS PAGE.

A second AS precipitation was then carried out as described on the chosen sample. The percentage AS before which the recombinant protein precipitates from the solution is the sample chosen, a higher range of AS percentages are used in the second cut to precipitate the recombinant protein from any remaining proteins in the sample.

The samples were dialysed after the second precipitation step and again a fraction is analysed by SDS PAGE.

When both AS percentages are chosen a large scale purification is carried out with 8 ml of partially purified recombinant antigen. The procedure is similar to that described above with larger volumes.

2.4.10 Western Blot Analysis of Recombinant Antigens

Solution and reagents as detailed in appendix section IV

1. An SDS-PAGE gel was run as described, with pre-stained markers. The sponges and 12 sheets of 3 MM filter paper were soaked in transfer buffer. The filters were cut to the size of the gel as was the nitrocellulose.

2. Two sponges were placed on each of the 4 grids and 3 pieces of filter paper on each of these. Each of the gels was put on one of the stacks of filters. The nitrocellulose which had been wet with transfer buffer was put on top of the gel. Ensuring there were no bubbles between any of the layers.

3. The second stack was placed on top of each of the membranes, and put in the blotting apparatus with the gel on the side of the negative (black) electrode and the nitrocellulose on the positive (red) side. The cooling water was set flowing through and the apparatus was connected to the power pack. The voltage was set constant at 30 volts for 1 hr, this was then increased to 60 volts for the second hour.

4. The apparatus was then taken apart and the nitrocellulose was placed in TBS briefly followed by blocking buffer for 1 hr. The membrane was then incubated in serum overnight at 4°C (The serum was diluted 1/100 in blocking buffer).

5. The filter was washed twice in TBST (0.1% (v/v) Tween-20) for 10 min and once in blocking buffer for 15 min. Meanwhile the secondary antibody was diluted 1/5000.
also in blocking buffer. This was an anti-human alkaline phosphatase conjugated antibody.

6 The filter was then incubated in secondary antibody for 1 hr at room temperature, followed by washing three times with TBST for 10 min each. In a clean container, BCIP/NBT substrate was used to cover the filter and the container was placed in the dark at room temperature for 30 min. The filter was then rinsed in distilled water to stop the reaction and placed in cling film to store.

2.4.11 Protein Purification using Phenyl Arsine Oxide (PAO) Agarose

Solution and reagents as detailed in appendix section V.

1 A 0.5 ml volume of PAO resin was packed into a 2.5 ml sterile syringe, which was plugged at the end with glass wool. The upper layer of 50% ethanol was removed. A cap was put on the end of the syringe.

2 The resin was resuspended in 1 ml of running buffer with 3 mM β-mercaptoethanol (β-mer), the resin was allowed to settle by gravity and the buffer was decanted by removing the cap and allowing the buffer to flow through.

3 A 2 ml volume of this buffer was then added to the column, and the column was allowed to rock gently for 1 hr at room temperature. Parafilm was used to seal the top of the column. This incubation was done to activate the column. The supernatant was then decanted as before.

4 The resin was resuspended in 1.5 ml of running buffer (without β-mer), again the resin was allowed to settle and the supernatant was decanted, this step was repeated twice.

5 A 1 ml volume of the cell lysate (containing the soluble recombinant protein and soluble E.coli proteins), was added to the column and rocked at room temperature for 1 hr.

6 The bottom of the column was opened and the lysate was allowed to flow through. This sample was collected in a microfuge tube and stored for SDS PAGE analysis.

7 The resin was washed twice with 1 ml running buffer with 0.25 mM β-mer, the flow through was collected and stored.

8 The recombinant protein was eluted using 1 ml running buffer with 500 mM β-mer, the flow through was collected and stored for analysis.
9 A 10 μl volume of each fraction was used for SDS PAGE analysis as described in section 2.4.6. The final eluate was assayed for protein concentration using the BCA protein assay as per manufacturer's instructions, PIERCE product no 23225.

10 The purified protein was stored in glycerol (15%), with leupeptin (2 μg/ml) and PMSF (17.4 mg/ml, 100 mM).

2.4.12 ELISA Analysis of Recombinant Antigens with Human Serum

Solution and reagents as detailed in appendix section VI.

1 Each protein was diluted to 10 μg/ml (recombinant protein, not total protein) in coating buffer, each well was coated with 100 μl of these solutions, the microtitre plate was incubated overnight at 4°C.

2 The coating solution was removed and 200 μl blocking buffer was added to each well, the plate was incubated for 2 hr at 37°C.

3 The blocking buffer was removed and 100 μl of serum was added to each well, and the plate was incubated for 2 hr at room temperature. (Sera were diluted 1/100 in TBS, 5% FCS, 0.05% NaN₃ and 0.05% Tween).

4 The serum was removed and the wells were washed six times with TBST (0.1% Tween). The secondary antibody [anti-human Ig-AP (1/5000), anti-human IgG-AP (1/6000), or anti-human IgM-AP (1/1000)] was diluted in blotto (5%).

5 A 100 μl volume of the diluted secondary antibody was added to each well and the plate was incubated for 1 hr at room temperature.

6 The secondary antibody was removed and the wells were washed six times with TBST (0.1% Tween), 200 μl of substrate was added, the plate was incubated for 30 min at room temperature in the dark (The substrate was prepared as per manufacturer's instructions - Sigma fast OPD substrate).

7 The optical density was measured at a wavelength of 414 nm.
2.5 Phage Display Libraries (NEB)
Solution and reagents as detailed in appendix section VII

2.5.1 ER2537 E.coli Strain Maintenance

ER2537 is an F-, recA- strain

All cultures for M13 propagation must be inoculated from colonies grown on media selective for presence of the F-factor. The F-factor of ER2537 contains a portion of the proline biosynthetic operon that complements a corresponding chromosomal deletion, so these cells can be selected by plating in the absence of exogenous proline, i.e. Minimal medium. Thiamine must be included in the medium as ER2537 is auxotrophic for thiamine.

ER2537 was streaked out from the glycerol stock provided onto a minimal plate, inverted and incubated at 37°C for 24 hr. The plate was stored wrapped in parafilm at 4°C for up to 1 month.

2.5.2 Phage Titering

1. A single colony of ER2537 was used to inoculate 10 ml of LB and incubated with shaking at 37°C until OD_{600nm} ~ 0.5.

2. Melted top agarose was dispensed into 3 ml aliquots and stored at 45°C until ready for use. One LB/IPTG/Xgal plate was prewarmed for each expected dilution. Tenfold serial dilutions of phage were made in LB, for amplified phage culture supernatants this was 10^8 - 10^{11}, for unamplified biopanning eluates the dilutions were 10^{-1} - 10^{4}.

3. When the culture had reached mid-log phase 200 μl was dispensed into microfuge tubes for each phage dilution. To these, 10 μl of each dilution was added, vortexed quickly and incubated at room temperature for 1-5 min.

4. The infected cells were transferred to a culture tube containing the top agarose, vortexed quickly and immediately poured onto a pre-warmed LB/IPTG/Xgal plate.

5. The plates were allowed to cool, inverted and incubated overnight at 37°C. Plaques were counted the following day.
2.5.3 Biopanning Procedure A

1. The monoclonal antibody 72A1 was diluted to 100 μg/ml in 0.1 M NaHCO₃ (pH 8.6), 1 ml of this solution was added to each plate (35 mm diameter), and swirled until the surface was completely wet. The plate was incubated overnight at 4°C with gentle agitation in a humidified container.

2. The next day 10 ml LB was inoculated with a single colony of ER2537, when amplifying the eluate on the same day a 20 ml culture was also inoculated with ER2537. Both cultures were incubated at 37°C with vigorous shaking.

3. The coating solution was poured off the plate and the plate was slapped face down to remove any residual solution. The plate was filled with blocking buffer and incubated for 1 hr at 4°C.

4. The blocking solution was then discarded and the plate was washed 6 times with TBST (0.1%(v/v) Tween-20). 4*10^10 phage were diluted with 1ml TBST (0.1%), this was added to the washed plate which was then rocked gently for 1hr at room temperature.

5. The non-binding phage were discarded by pouring off and slapping the plate face down, the plate was then washed 10 times with TBST(0.1%). (A clean section of paper towel was used at each wash.)

6. The bound phage were eluted with 1ml of elution buffer (0.2 M glycine-HCl pH2.2, 1 mg/ml BSA), the plate was rocked gently for 10 min at room temperature, the eluate was pipetted into a microfuge tube and neutralised with 150 μl 1 M Tris-HCl (pH9.1), 1 μl of the eluate was titered as described above.

7. The remainder was amplified, as follows. The eluate was added to 20 ml ER2537 culture at OD₆₀₀nm~0.5, incubated at 37°C with vigorous shaking for 4.5 hr.

8. The culture was transferred to a centrifuge tube and centrifuged for 10 min at 11950 g at 4°C, the supernatant was transferred to a fresh tube and re-centrifuged for 2 min. The upper 80% of the supernatant was pipetted to a fresh tube to which 1/6 volume of PEG/NaCl was added. The phage were allowed to precipitate at 4°C for 1 hr, or overnight.

9. The PEG precipitation was centrifuged for 15 min at 11950 g at 4°C, the supernatant was decanted and the tube was re-spun briefly the residual supernatant was removed.

10. The pellet was resuspended in 1ml TBS, this solution was microcentrifuged for 5 min at 4°C, to pellet residual cells. The supernatant was re-precipitated with 1/6 volume
of PEG/NaCl by incubation on ice for 15-60 min. The tube was microcentrifuged for 10 min at 4°C, the supernatant was discarded and the tube was re-spun briefly to remove residual supernatant.

11 The pellet was resuspended in 200 μl TBS, 0.02% NaN₃, microcentrifuged for 1 min and the supernatant transferred to a fresh tube. This amplified elute was titered as described above. A plate was coated as before for the second round of panning.

12 The blue plaques which resulted after titering were counted and the phage titer was determined. This value was used to calculate the phage input volume required for the second round. 1-2×10¹¹ pfu were required.

13 The second and third round of biopanning were carried out as for the first round, the Tween-20 concentration was raised to 0.5%(v/v) for all washes and dilutions in these rounds.

14 When the third round unamplified eluate was titered the blue plaques were selected for phage purification and sequencing.

2.5.4 Plaque Amplification

1 An overnight culture of ER2537 in LB was incubated at 37°C with vigorous shaking.

2 This culture was diluted 1/100 in LB, dispensed in 1 ml aliquots into culture tubes, and inoculated with a single blue plaque picked from a third round titer plate. (The plates should not be incubated for longer than 18 hr as deletions may occur.) The tubes were incubated at 37°C for 4.5-5 hr with vigorous shaking.

3 The culture was then transferred to a microfuge tube and centrifuged for 30 s at 13000 rpm, the supernatant was removed to a fresh tube and recentrifuged, the upper 80% of this supernatant was transferred to a fresh tube.

2.5.5 Rapid Purification of Sequencing Template

1 To prepare template for sequencing, 750 μl of the phage-containing supernatant was transferred to a fresh microfuge tube.

2 To this 300 μl of PEG/NaCl was added, mixed by inversion and let stand at room temperature for 10 min. This was then centrifuged for 10 min at 13000 rpm and the supernatant removed.
3 The pellet was thoroughly resuspended in 100 µl of iodide buffer and 250 µl of 100% ethanol was added. After incubation for 10 min at room temperature the solution was centrifuged for 10 min at 13000 rpm.

4 The supernatant was discarded and the pellet was washed with 70% ethanol, centrifuged as before for 2 min. The supernatant was removed and the pellet was allowed to dry.

5 The pellet was resuspended in 20 µl of sterile TE pH 8.0. 10 µl of this template was used for manual Sanger dideoxy sequencing with $^{35}$S.

2.5.6 **Biopanning Procedure B (solution binding with Protein G capture)**

1. A single colony of ER2537 was used to inoculate 10 ml LB, when amplifying the eluate on the same day a 20 ml culture was also inoculated with ER2537. Both cultures were incubated at 37°C with vigorous shaking.

2. A 75 µl volume of protein A (50% aqueous suspension) was pipetted into a microfuge tube, to this 1 ml TBST (0.1% Tween-20) was added. The resin was suspended by gently tapping the tube, the resin was the pelleted by centrifugation at low speed for 30 s - 1 min.

3. The supernatant was removed and the pellet was resuspended in 1 ml blocking buffer, incubated at 4°C for 1 hr. The solution was mixed every 10 min to keep the resin in suspension.

4. Four*10$^{10}$ phage and 300 ng of antibody (72A1) were mixed together to a final volume of 200 µl of with TBST (0.1%), and incubated at room temperature for 20 min.

5. The resin was pelleted by centrifugation and washed 4 times with TBST (0.1%), pelleting each time by centrifugation for 1 min. The phage-antibody mix was added to the resin, mixed gently and incubated at room temperature for 15 min, the contents were mixed occasionally.

6. The resin was pelleted, the supernatant discarded, and the resin was washed 10 times with 1 ml TBST (0.1%). The bound phage were eluted by suspending the pellet in 1 ml elution buffer and incubating for 10 min at room temperature.

7. This mixture was centrifuged for 1 min at 13000 rpm and the supernatant was carefully to a fresh tube, this was then neutralised with 150 µl 1 M Tris-HCl, pH 9.1.
One microlitre of the eluate was titered as described in section II(ii) and the remainder was amplified as described for procedure A.

The second and third round of biopanning was carried out as above with the Tween-20 concentration raised to 0.5%(v/v) for all washes and dilutions.

Plaque amplification and purification of sequencing templates was carried out as described above.

### 2.5.7 Preparation of Phage for Assay via ELISA and DOT Blot

1. A single colony of ER2537 was used to inoculate 5 ml LB and incubated at 37°C overnight with vigorous shaking. This culture was diluted 1/100 in 20 ml of LB for each clone to be characterised.

2. Five microlitres of phage supernatant (from the plaque amplification procedure) was added to each culture and incubated at 37°C with vigorous shaking for 4.5 hr. The culture was transferred to a centrifuge tube and spun for 10 min at 11950 g at 4°C, the supernatant was transferred to a fresh tube and re-centrifuged briefly.

3. The upper 18 ml was removed to a fresh tube and 3 ml PEG/NaCl was added to precipitate the phage. This was done at 4°C for 1 hr, or overnight. This solution was then centrifuged for 15 min at 11950 g at 4°C, the supernatant was removed and the tube was re-spun briefly, the residual supernatant was removed.

4. The pellet was then resuspended in 1 ml TBS and transferred to a microfuge tube and spun for 5 min at 4°C. The supernatant was re-precipitated in a fresh tube with 1/6 volume PEG/NaCl incubated on ice for 15 min.

5. The solution was centrifuged for 10 min at 4°C, the supernatant was discarded, the tube was re-spun briefly and the residual supernatant removed.

6. The pellet was resuspended in 50 μl TBS and titered as described previously.

### 2.5.8 Phage ELISA

1. The selector antibody, 72A1, was diluted to 10 μg/ml in 0.1 M NaHCO₃ (pH 8.6), 200 μl aliquots were loaded into the appropriate number of wells on a 96 well titer plate (Costar microtiter plate). The plate was incubated overnight at 4°C with agitation in a humidified container.

2. The following day the coating solution was removed and the wells were blocked with 200 μl of blocking buffer (PBS 2% marvel) for 2 hr at 37°C.
3 Each phage preparation was diluted to 100 μl with PBS and added to the ELISA plate. The plate was incubated at room temperature for 2 hr. This solution was then removed and the wells were washed 6 times with TBST (0.5% Tween-20).

4 The primary antibody, rabbit anti-bacterophage antibody, was diluted 1/1000 in blocking buffer, 200 μl of this solution was added to each of the wells and the plate was incubated for 2 hr at room temperature.

5 The wells were then washed 6 times with TBST as before, the secondary antibody, anti-rabbit-alkaline phosphatase conjugate, was diluted 1/1500 in blocking buffer, 200 μl of this solution was added to each of the wells. The plate was incubated at room temperature for 1 hr, and the washing step was repeated.

6 The substrate pNPP was prepared as per manufacturers instructions, 100 μl of this substrate was added to each well and the plate was incubated in the dark for 30 min at room temperature.

7 Where the solution became yellow a positive result was recorded, if no colour change occurred a negative result was recorded, the colour intensity was not recorded.

### 2.5.9 Phage DOT Blot

1 Phage were prepared as for ELISA, and the volume of each sample was made up to 200 μl with sterile PBS. A DOT blotting apparatus was used, this was connected to a vacuum pump.

2 The nitrocellulose was cut to the size of the blotter and placed between the perforated mat and the cover of the blotter. A vacuum was drawn and the blotter was tightened. The vacuum was turned off and the samples were loaded into the wells, allowed to stand for 1 hr and then a vacuum was drawn for 1 hr.

3 The nitrocellulose was removed and placed in a clean tray to which blocking buffer (as for plaque lift) was added. This was incubated with agitation for 1 hr at room temperature, then washed for 10 min with TNT A, 10 min with TNT B and 10 min with TNT A, constantly agitating.

4 The primary antibody (72A1) was diluted 1/1000 to a concentration of 1 μg/ml in blocking buffer with sodium azide (0.05% NaN₃). The nitrocellulose was covered with this solution and incubated for 1 hr at room temperature with agitation.
5 The filter was then washed as before with TNT A and TNT B. The secondary antibody (anti-mouse-POD conjugate) was diluted 1/750 in blocking buffer without sodium azide, the filter was covered with this solution and incubated for 1 hr at room temperature with agitation.

6 The filter was washed again as before and then covered with substrate (Supersignal® substrate PIERCE product no 34080, prepared as per manufacturer’s instructions) and incubated for 10 min.

7 The filter was wrapped in clingfilm taking care to exclude any air bubbles, exposed to X-ray film for 30 s, 1 min and overnight. The film was developed as described in section 2.7.4.

2.5.10 Deglycosylation of gp350/220

1 A 100 μl aliquot of gp350/220 (concentration unknown) was placed in a microcentrifuge tube, 1 μl of 10% SDS was added, the tube was vortexed and centrifuged briefly. This mix was incubated for 10 min at 100°C to denature.

2 After boiling the tube was centrifuged briefly and 1 μl of NP40 was added, the tube was vortexed briefly and centrifuged.

3 Two tubes were prepared with the following reagents, A as a control (no deglycosylation enzymes), B with deglycosylases:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>A(μl)</th>
<th>B(μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-glycosidase</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>N-glycosidase</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>PMSF (17.4 mg/ml)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>gp350/220 (denatured)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Deglycosylation buffer</td>
<td>65</td>
<td>54</td>
</tr>
<tr>
<td>Total volume</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

4 Each reaction tube was vortexed briefly and centrifuged, the reaction mixes were incubated for 18 hr at 37°C.

5 The tubes were centrifuged and 10 μl aliquots were taken for analysis on SDS PAGE.
followed by Western blot analysis, as described in sections 2.4.6 and 2.4.8. The remainder was stored at 4°C.

2.6 Protocols for the 15mer Phage Library

Solution and reagents as detailed in appendix section VIII. The 15mer library displays a 15mer peptide on each of the 5 pIII proteins of the fUSE5 filamentous phage. The library consists of $1 \times 10^{12}$ clones and the concentration of phage is $1 \times 10^{14}$ virion/ml. This library was a gift from Dr. Conley.

2.6.1 Bacterial Strains

_E. coli_ K91 and K91KAN.

K91 and K91KAN are standard _E. coli_ strains for propagating most filamentous phage strains. They have ~5 F pilin/cell (as opposed to 0.5/cell in most male strains) and give excellent, large plaques with wild-type filamentous phage. K91KAN also carries the kanamycin resistance gene. Both strains are grown on LB agar with or without kanamycin as appropriate.

2.6.2 Preparation of Terrific Broth Cultures

A single colony of K91KAN cells was used to inoculate 5 ml NZY (100 μg/ml kanamycin) and incubated at 37°C with vigorous agitation overnight. Of this culture 100 μl was used to inoculate 10 ml Terrific broth in a 125 ml flask. The culture was incubated at 37°C with vigorous shaking. When the O.D. of 1/10 dilution ~ 0.1-0.2 the shaking was slowed down to allow any sheared F pilin to regenerate. The cells are then ready for use within an hour.

2.6.3 Titering of Plaque Forming Units (pfu)

1. A single colony of K91KAN was used to inoculate 10 ml NZY (kan), the culture was incubated at 37°C with vigorous shaking until the O.D. ~ 0.2 (~4hr).
2. The TB soft agar was melted and 3 ml aliquots were put in glass tubes, these were stored in an oven at 55°C.
3. The phage were diluted appropriately in TBS/gelatin and 400μl of the K91KAN culture was added to each dilution. This solution was mixed and added to a tube of
soft-agar, the tube was vortexed and immediately poured onto an NZY(kan) plate with 40 µg/ml tetracycline and 100 µg/ml kanamycin.

4 The soft agar was spread evenly over the plate, the agar was allowed to solidify and the plates were incubated at 37°C overnight.

2.6.4 Titering of Transducing Units (TUs)

1 Terrific broth cultures of K91KAN were prepared as described in section 2.6.2, and the eluate or amplified phage were diluted with TBS/gelatin. A range of dilutions were covered to ensure a particle concentration of $3 \times 10^5$ virions/ml - $3 \times 10^6$ virions/ml.

2 Ten microlitres of each dilution was placed in a sterile 10 ml tube, to each of these samples 10 µl of the K91KAN terrific broth culture was added. The two were mixed thoroughly and incubated at room temperature for 10 min to allow the phage to infect the concentrated cells.

3 After incubation 1 ml of NZY (0.2 µg/ml tetracycline) was added to each tube and they were incubated at 37°C for 40 min with agitation.

4 Two hundred microlitre aliquots of the infected cells were then spread on NZY plates containing 40 µg/ml tetracycline and 100 µg/ml kanamycin. The plates were incubated overnight at 37°C.

2.6.5 Biopanning

1 Forty micrograms of the monoclonal antibody (72A1) was added to a siliconised microfuge tube, 44 µl of 1 M NaHCO3 was added to this, and the volume was made up to 24 µl with sterile distilled water.

2 One milligram of biotin-NHS was dissolved in 2 ml 2 mM NaOAc (pH6), 20 µl of this solution was added to the monoclonal antibody solution and incubated for 2 hr at room temperature.

3 Five hundred microlitres 1 M ethanolamine was added and incubated for 2 hr at room temperature (the ethanolamine reacts with any residual biotin). Twenty microlitres 50 mg/ml dialysed BSA and 1 ml TBS were added, and the mix was concentrated on a Centricon ultrafilter (Amicon) with a 30-kD cut-off.

107
The retentate was washed twice with 2 ml TBS and once with TBS/0.02% NaN3. The retentate was then collected and stored in a 0.5 ml microfuge tube and stored at 4°C. The volume was measured and the concentration of biotinylated antibody was calculated.

Nine hundred microlitres of distilled water and 100 μl 1 M NaHCO3 were added to a petri dish (35 mm diameter), 10 μg streptavidin was added and the plate was agitated to mix the contents. The plate was put in a humidified box and incubated overnight at 4°C with agitation.

This solution was then discarded and replaced with blocking buffer, the plate was incubated for at least 1 hr at 4°C. The blocking buffer was then returned to its container (can be re-used until microbial growth becomes evident) and stored at 4°C, the plate was washed 6 times with TBS/Tween.

Five micrograms of biotinylated antibody was added to the dish, and it was allowed to rock at 4°C for 2 hr, in a humidified container. Four microlitres of 10 mM biotin-NHS (filter-sterilised) was added to the plate and it was allowed to rock for a further hour at 4°C.

The dish was then washed 6 times with TBS/Tween, then 400 μl TBS/Tween was added to the plate and 4 μl 10 mM biotin-NHS was added. Five microlitres of the original 15mer library was added to this solution. The dish was incubated at 4°C in a humidified container for 4 hr.

The phage were poured out of the dish, and the dish was slapped face down to remove any residual liquid. The dish was washed 10 times with TBS/Tween, a clean paper towel was used after each wash.

Four hundred microlitres of elution buffer was added to the dish, and incubated at room temperatures for 10 min. The eluate was pipetted into a microfuge tube containing 75 μl 1M Tris HCl pH 9.1. One microlitre of the eluate was used for titration and the remainder was amplified in K91KAN E. coli.

The first eluate was concentrated on a Centricon 30-kD ultrafilter and washed once with TBS. The retentate was back centrifuged into a collection tube and transferred to a microfuge tube. A further 50 μl of TBS was added to the membrane to wash, again this was back centrifuged and transferred to the microfuge tube giving a final volume of 100 μl. (This step was not necessary for the second and third eluate.)
12 K91Kan terrific broth cells were prepared as described above, 100 µl of these cells were mixed with 100 µl of eluate, the solution was stirred gently with a pipette tip and allowed to react for 10-30 min at room temperature. This was then added to 20 ml of pre-warmed NZY medium with 0.2 µg/ml tetracycline in a 125 ml flask.

13 The culture was incubated at 37°C for 30-60 min, 20 µl of 20 mg/ml tetracycline was added to the flask (20 µl was removed at this point) and the culture was incubated at 37°C with vigorous shaking overnight.

14 The 20 µl sample taken was diluted 10⁻¹ for the first eluate and 10⁻¹ and 10⁻² for the second and third eluate, and was subsequently plated on NZYtet,kan. The plates were incubated overnight at 37°C, and the colonies were counted on the following day.

15 The 20 ml overnight culture was centrifuged at 2987 g for 10 min at 4°C, the supernatant was removed to a clean tube and re-centrifuged at 11950 g for 10 min at 4°C. This supernatant was added to a tube with 3 ml PEG/NaCl, the contents were mixed by inverting ~100 times and was incubated at 4°C for at least 4 hr, or overnight.

16 This solution was then centrifuged at 11950 g for 15 min at 4°C, the supernatant was discarded and the centrifugation was repeated, any remaining fluid was then removed. The pellet was then resuspended in 1 ml TBS, the tube was centrifuged briefly and the solution was transferred to a microfuge tube which was centrifuged for 1 min to remove any insoluble matter.

17 The supernatant was added to a tube with 150 µl PEG/NaCl, mixed by ~100 inversions, and incubated at 4°C for at least 1 hr. The tube was centrifuged at high speed for 10 min at room temperature, the supernatant was removed and the tube recentrifuged, the remaining fluid was removed.

18 The phage pellet was resuspended in 200 µl TBS (0.02% NaN₃). The tube was centrifuged for 1 min to remove any insoluble matter and the supernatant was transferred to a fresh tube.

19 For the second and third round of biopanning the antibody (72A1) was pre-reacted with the phage and then panned on the streptavidin coated plate. The petri dish was coated as before with streptavidin. 100 µl of phage from the amplified eluate was added to 100 nmoles of the biotinylated MAb in a microfuge tube, this mixture was allowed to react overnight at 4°C.
20 Four hundred microlitres of TBS/Tween was added to the phage/antibody solution and immediately added to the streptavidin coated dish (had been blocked and washed). The dish was allowed to rock gently for 10 min at room temperature.

21 The washing and elution were carried out as described for round one. This was followed by titrating and amplification of the second eluate. The procedure for round three was similar to that for round two but the amount of biotinylated antibody used was reduced to 0.1 nM.

2.6.6 Small-scale Propagation and Processing of Phage

1 NZY with 20 mg/ml tetracycline was aliquoted in 1.7 ml portions into culture tubes. Single colonies were isolated from a third round titer plate and used to inoculate the media, these cultures were incubated overnight with vigorous shaking at 37°C.

2 The cultures were poured into a microfuge tube and centrifuged for 30 s to pellet the cells. 1 ml of the supernatant was transferred to a fresh tube with 150 μl PEG/NaCl. The solution was mixed by ~100 inversions and incubated at 4°C for at least 4 hr, or overnight.

3 This was then centrifuged at 13000 rpm for 15 min at 4°C, the supernatant was discarded and the tube was recentrifuged and any residual fluid was removed.

4 The pellet was dissolved in 500 μl TBS by vigorous vortexing.

2.6.7 Preparation of Sequencing Template

1 To 200 μl of phage (prepared as in section III(iv)), 200 μl of phenol was added, the tube was vortexed for 20 s, allowed to stand for 20 min, vortexed for a further 20 s and centrifuged for 5 min at 13000 rpm.

2 The upper aqueous phase was removed to a fresh tube, an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added, the tube was vortexed for 20 s and centrifuged for 5 min at 13000 rpm.

3 Again the upper aqueous phase was removed to a fresh tube, an equal volume of chloroform/isoamylalcohol (24:1) was added. The tube was vortexed for 20 s and centrifuged for 5 min at 13000 rpm, the upper aqueous phase (~150 μl) was removed to a tube containing 250 μl TE.
Forty microlitres of 3 M NaOAc and 1 ml ethanol were added, and the tube was incubated for 1 hr on ice to allow the DNA to precipitate. The tube was centrifuged for 30 min at 13000 rpm, the supernatant was removed and the pellet was washed with 1 ml 70% ethanol.

The tube was centrifuged for 5 min at 13000 rpm, the supernatant was removed and the tube was recentrifuged. Remaining fluid was removed and the pellet was resuspended in 7 μl sterile distilled water.

### 2.6.8 Immunological Screening of Plaques or Colonies

1. Plates with either plaque forming units or transducing units were incubated at 37°C overnight. If colonies were being screened, the plates were placed at 4°C for 2 hr before lifting.

2. Nitrocellulose membrane was cut to the size of the petri dish and given orientation marks corresponding to marks on the plates. The filter was placed face down on the colonies or plaques, then lifted straight off and placed in a 1 L beaker of TNT (7.5 ml/filter).

3. BSA, gp350/220, 72A1 and the secondary antibody were all dotted on to a piece of nitrocellulose and washed and incubated as for the plaque/colony lift. BSA was the negative control, gp350/220, 72A1 and the secondary antibody were all positive controls. A range of concentrations were used.

4. The beaker was covered with cling film and agitated for 30 min at room temperature. The TNT was replaced by a fresh aliquot and incubated for a further 30 min.

5. The wash was then removed and blocking buffer (with NaN₃) was added, the beaker was agitated at room temperature for 30 min.

6. The primary antibody (72A1) was diluted to 0.45 μg/ml in blocking buffer (with NaN₃), the blocking buffer was poured off and the primary antibody was added (7.5 ml/filter). The filters were incubated in this solution for 2 hr at room temperature with agitation.

7. The filters were then washed 3 times for 10 min each with TNTA, TNTB, and TNTA. The secondary antibody (anti-mouse alkaline phosphatase conjugate) was diluted 1/5000 with blocking buffer (without NaN₃) (7.5 ml/filter). This was incubated for 1 hr at room temperature with agitation.
The filters were washed as before and each was placed in a clean container. Each filter was covered with BCIP/NBT substrate and incubated in the dark for 30 min, then analysed for positive reactions which appear as purple spots on the filter.

2.7 Sequencing (T7 Sequencing Kit- Pharmacia Biotech)

Solution and reagents as detailed in appendix section IX

Notes
(1) The heating block was set at 60°C
(2) 1.5 ml microfuge tubes were used for initial primer annealing and labelling
(3) 0.5 ml tubes were used for termination reactions
(3) S35 was taken from the freezer 30 min before use

Dilutions of T7 DNA polymerase

<table>
<thead>
<tr>
<th>no of templates</th>
<th>polymerase</th>
<th>dilution buffer(μl)</th>
<th>total volume(μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>6</td>
<td>7.5</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>10</td>
<td>12.5</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>3.5</td>
<td>14</td>
<td>17.5</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>4.5</td>
<td>18</td>
<td>22.5</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>

The primer was diluted to 2.5 μM

The template was prepared as in section 2.5.5 or section 2.6.6

2.7.1 Sequencing Reactions

To 10 μl of template (phage DNA), 2 μl of diluted primer was added in a microfuge tube, 2 μl of annealing buffer was then added, the tube was vortexed, centrifuged briefly, then incubated at 60°C for 10 min and allowed to stand at room temperature for at least 10 min, then centrifuged briefly.
2 To this mix, 3 μl labeling mix (dATP), 1 μl labeled dATP (S<sub>33</sub>) and 2 μl diluted T7 DNA polymerase were added. These were mixed gently by pipetting and incubated at room temperature for 5 min.

3 Four tubes were labeled A,C,G,T for each template, 2 5 μl of each of the “read short mixes” were added to their corresponding tube and incubated for 1 min at 37°C.

4 To each of the 4 pre-warmed sequencing mixes, 4 5 μl of the labeling reaction was added, mixed by gentle pipetting, and incubated at 37°C for 5 min.

5 Five microlitres of stop solution was added and mixed gently.

6 Four microlitres of each reaction was added to a fresh tube, incubated at 75-80°C for 2 min, and immediately loaded on the sequencing gel. The remainder of the unheated reactions was stored at -20°C.

2.7.2 Preparation of the Sequencing Apparatus

The plates were washed in detergent to remove all traces of gel, rinsed thoroughly with tap water followed by ultra pure water, wiped dry with clean dry tissue removing all tissue and dust. 10% SDS was poured onto each plate and rubbed vigorously with clean tissue, another clean dry tissue was used to remove the detergent. 100% ethanol was poured onto the plates and wiped in one direction along the entire length of the plate until dry again. The large plate was siliconised using “sigmacote”, of which a few drops were applied at one end and wiped along the plate in one direction. The plates were then clamped together with the spacers along each side.

2.7.3 Casting the Gel

1 Starting with a clean dry casting tray a paper sealing strip was placed into the casting tray. To 20 ml of gel mix, 250 μl 10% APS and 100 μl TEMED was added. This gel solution was poured quickly onto the sealing strip and the plates were placed on top and clamped into the tray. When set (tip to check), the main gel was poured at 45° angle using a 50 ml syringe slowly and constantly.

2 The comb was inserted blunt end first and the gel was allow to set, at least 3 hrs. Two litres of 1x TBE was prepared from the 10x stock with ultra pure water. The comb was removed and inserted with the sharp tooth edge toward the gel, the wells were washed thoroughly with a syringe and fine needle. Four hundred millilitres of buffer was poured in the bottom reservoir and the IPC chamber was filled. The gel was
prerun for 45 min at least, using sample dye in a few wells to ensure the samples would run correctly

3 After a gradual increase of power over a 10 min period the gel was run at 1700 volts for 3 25 hr for the Conley library, and 2 75 hr for the NEB library (due to the required length of the sequence to be read)

2.7.4 Drying and Developing

1 The plates were separated and 3MM filter paper (cut to size) was placed on top of the gel, avoiding bubbles. The paper was lifted gently taking the gel with the paper. This was then covered with cling film and placed on top of another sheet of 3MM filter paper in the gel dryer, with the cling film facing up.

2 The gel was dried at 80°C for 1-2 hr. When dry the gel was placed in a cassette and in the dark room the cling film was removed and a sheet of X-ray film was placed on the gel. The cassette was sealed shut and the gel was exposed to the film for 48 hr.

3 The film was removed from the cassette in the dark, placed in developer for 5 min, water for 1 min, and fixer for 3 min. The film was then dried for ~ 30 s (when developing, the film was agitated continuously in each of the solutions.)
Chapter 3

Development of recombinant EBV antigens as reagents for serodiagnosis of EBV-related disease.
3.1 Introduction

Serological testing is of paramount importance in the diagnosis of EBV-related disease. As discussed in section 1.5, the Paul-Bunnell heterophile antibody test has been the method of predominant use to date (Paul and Bunnell, 1932, Elgh and Linderholm, 1996). A number of ELISA methods have been developed, mainly using cell-culture derived antigens. The performance of these diagnostic kits varies (Weber et al., 1996, Gutierrez et al., 1997), (see section 1.5.3), and large-scale production of antigens from cell culture is expensive. Expression of EBV antigens in *Escherichia coli* would allow the large-scale production of recombinant antigens in large quantities at a lower cost. The Thiofusion expression system was chosen (see section 1.8) as it permits the high level expression of soluble recombinant protein, and provides a convenient method of purification (Lunn et al., 1982 and 1984, LaVallie et al., 1992).

Three EBV antigens were chosen for expression, EBNA1, p18VCA and diffuse early antigen (EA-D) (sections 1.3.2, 1.3.3, 1.5.1). The BARFO ORF was also cloned, and the gene product expressed in *E. coli*. The antibody profile of an individual (IgG and IgM), to these antigens, is an excellent indication of the type of EBV infection the individual has at a particular time (discussed in section 1.5.1, see table 4).

In summary, the open reading frame (ORF) DNA sequence encoding each antigen was cloned into the *E. coli* expression vector pTrxFus, and the recombinant proteins were expressed as fusions with thioredoxin. These proteins were partially purified and analysed for reactivity against a panel of serum by Western blot and ELISA. The sera tested had been previously characterised as IM-positive, VCA-positive or -negative, EBNA-positive or -negative, CMV-positive, EBV-positive or normal, by alternative methods.

The sera were partially characterised using the novel antigens by ELISA, and compared with the profiles determined by alternative commercially available diagnostic kits. The assays used were as follows.
• BIOTEST, Anti-EBV Recombinant EBNA IgG ELISA
• Gull, EB VCA IgM ELISA
• Baxter Bartels, CMV IgM EIA and EBV IgM EIA
• ORGANON TEKNIKA, Monosticon Dri-DOT test (heterophile antibody test)
• Gull, Anti-VCA IgM IFA

3.2 Sub-cloning of Antigen coding DNA sequence

3.2.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was used to specifically amplify each ORF from the cDNA previously cloned into either a pGEM7zf vector (BKRF1), or the pBR322 vector (BFRF3 and BARFO). The EA-D gene BMRF1 was amplified from genomic DNA isolated from the Raji cell line (Burkitt Lymphoma EBV-positive cell line). The PCR primers were designed to incorporate a restriction enzyme site at the 5’end, to aid sub-cloning into the pTrxFus vector. In this regard, BamH1 was chosen for each cloning experiment as using this site simplified the in-frame cloning of each PCR product and obviated the need for a double digestion. The PCR program for each amplification was as follows (Table 5), the single variable was the annealing temperature (Ta), which was determined from Tm, the melting temperature of each primer [Tm = 2(T+A) + 4(G+C)]

<table>
<thead>
<tr>
<th>Stage no.</th>
<th>Step no.</th>
<th>Temp. (°C)</th>
<th>Time (min)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>94</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Ta</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>72</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>72</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>15</td>
<td>Hold</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5: Polymerase Chain Reaction (PCR), standard program, only Ta was varied for each antigen, depending on the primers.
Primers were designed incorporating a BamHI site at the 5' end of each, the forward primer was designed such that a truncated form of EBNA1 was amplified from the BKRF1 ORF in p7CMVE1, which contains the complete EBNA1 ORF. Figure 12 shows a schematic representation of the EBNA1 protein and the gly-ala repeat which is excluded from the truncated protein. The primers were designed so that the cloned product was in the correct translational reading frame with the thioredoxin gene of pTrxFus. The annealing temperature was 61°C. The truncated form, 829 bases, encodes a protein approximately 29 kd which in fusion with thioredoxin forms a protein 41 kd in size. The deleted portion of BKRF1 has homology to cellular DNA (Heller et al., 1982), antibodies to the protein encoded by this part of the gene also react with cellular proteins (Dillner et al., 1984; Luka et al., 1984).

EBNA1

Figure 12: Schematic representation of the EBNA1 protein.

Forward primer
5' CGG GAT CCC AGG AGT CCC AGT AGT CAG TCA 3'

Reverse primer
5' CGG GAT CCT AAT ACG ATT GAG GGC GTC TCC 3'

BamH1 sites underlined above.

Following PCR, an amplified product of the expected size was detected and there were no non-specific amplified products. (Figure 13)
Figure 13: Amplification of 829 bases of the BKRF1 ORF by PCR. PCR products were electrophoresed through a 1% agarose gel. Lane1- DNA ladder, lanes 2, 3 and 4 - 3 μl of a 50 μl PCR reaction, lane 5 - PCR negative control (no template)

3.2.1.2 p18VCA
The viral capsid antigen, p18VCA, is encoded by the EBV BFRF3 ORF, which was present on a larger genomic BamHI DNA fragment cloned into pBR322, pBRBamHI F Primers were designed to amplify the complete p18VCA gene, again BamHI sites were incorporated into the primers to facilitate cloning into the BamHI site of pTrxFuse The first codon of BFRF3 was changed from a methionine to aspartic acid (GAC) so as to remove the possibility of internal translation initiation during the rProtein production. An annealing temperature of 44°C was used The amplified product was 528 bases and encoded an 18 kd protein which when fused with thioredoxin was 30 kd

Forward primer
5’ GTT ATG GAT CCC CGG CTG CCC AAG 3’

Reverse primer
5’ GTT TCG GAT CCT CTA CTG TTT CTT 3’

BamHI sites are underlined

Following PCR, an amplified product of the expected size was detected and there were no non-specific amplified products (Figure 14)
Figure 14: Amplification of the BFRF3 ORF by PCR. PCR products were electrophoresed through a 1% agarose gel. Lane 2 - DNA ladder, lanes 1, 3, and 4 - 3 μl of a 50 μl PCR reaction.

3.2.1.3 Early antigen-D

The gene for the EA-D protein BMRF1 was amplified by PCR from purified genomic Raji DNA. The primers have a BamH1 site incorporated and were designed to permit amplification of the whole BMRF1 ORF. The first codon of BMRF1 was changed from a methionine to an aspartic acid (GAC) so as to remove the possibility of internal translation initiation during the rProtein production. The annealing temperature used was 65°C. The PCR product was 1234 bases in length and encoded a 44 kd protein, the fusion protein was predicted to be 56 kd in size.

Forward primer
5' CGG GAT CCC GAA ACC ACT CAG ACT CTC 3'

Reverse primer
5' CGG GAT CCG GAA ACC ACT CAG ACT CTC 3'

BamH1 sites are underlined.

Following PCR, an amplified product of the expected size was detected (Figure 15), there was another non-specific product amplified at approximately 2.4 Kb. The complete PCR
reaction was run on a low melt agarose gel and the required fragment, BMRF1 was excised for purification by the QIAEX II agarose gel extraction procedure (sections 2.3.8 and 2.3.9), therefore no nonspecific fragments were present during ligation

![Amplification of the BMRF1 ORF by PCR](image_url)

**Figure 15:** Amplification of the BMRF1 ORF by PCR. PCR products were electrophoresed through a 1% agarose gel. Lanes 1 - DNA ladder, lanes 2, 3, 4 and 5 - 3 μl of a 50 μl PCR reaction

### 3.2.1.4 BARF0

The BARF0 primers allowed amplification of the entire BARF0 ORF from pBRBamHI A, a pBR322 plasmid with the EBV BamHI A fragment cloned. The first codon of BARF0 was changed from methionine to aspartic acid (GAC) so as to remove the possibility of internal translation initiation during the rProtein production. The annealing temperature was 45°C. The PCR product was 1479 bases in length and encoded a 52 kd protein, 64 kd when fused with thioredoxin. A BamHI site was incorporated to aid cloning, as for each of the genes amplified.

**Forward primer**

5' TGC CAG TGG GAT CCC GGG TAC GCT 3'

**Reverse primer**

5' TAC GGG GAT CCT AAA GTC GAT GTA 3'

BamHI sites are underlined.

Following PCR, an amplified product of the expected size was detected and there were no non-specific amplified products (Figure 16)
Figure 16: Amplification of the BARF0 ORF by PCR. PCR products were electrophoresed through a 1% agarose gel. Lanes 1, 2, and 3 - 3 µl of a 50 µl PCR reaction, lane 4 - DNA ladder.
3.2.2 Ligation and Transformation

Using the Thiofusion system the cloning plasmid pTrxFus was used, figures 17 and 18 show a schematic representation of the cloning vector pTrxFus and the multiple cloning site of the plasmid.

Figure 17: Schematic representation of the pTrxFus plasmid.

- ampicillin ORF: bases 201-1061
- ColE1 origin: bases ~1300-1880
- $P_l$ promoter: bases 2159-2187
- ribosome binding site: bases 2709-2726
- thioredoxin ORF: bases 2724-3050
- enterokinase cleavage site: bases 3051-3080
- multiple cloning site: bases 3081-3112
- aspA transcription terminator: bases 3113-3179
SmaI  
KpnI  
BamHI  
XbaI  
SalI  
PstI

5' GAT GAC GAT GAC AAG GTA CCC GGG GAT CCT CTA GAG TCG ACC TGC AGT 3' 
3' CTACTG CTACTGTTC CAT GGG CCC CTA GGA GAT CTC AGC TGG ACG TCA 5'

Asp Asp Asp Asp Lys Val Pro Gly Asp Pro Leu Glu Ser Thr Cys Ser

**Enterokinase cleavage site**

Figure 18: The multiple cloning site of pTrxFus. The base pair sequence is presented in groups of three so as to highlight the translational ORF which runs through the polylinker.

Reaction mixes were extracted once with phenol/chloroform and the DNA was precipitated with ammonium acetate and isopropanol (sections 2.3.3 and 2.3.5). The purified DNA fragments were analysed on 1% agarose gels to ensure that the product had been recovered after the procedure and was suitable for restriction digestion and cloning. The purified PCR product and the vector DNA were digested with BamHI as described in section 2.3.11, cleaned by phenol/chloroform extraction and precipitated with sodium acetate and ethanol, as described in sections 2.3.3 and 2.3.4.

Phosphatase treatment of the vector DNA was carried out after restriction with BamHI to prevent re-circularisation of the plasmid (section 2.3.12). The ligation reactions were performed as described in section 2.3.13. As the concentration of each was not determined exactly after digestion and purification, the ratio of vector DNA to insert DNA was varied for a number of reactions. Control reactions were also performed to ensure the ligation and the phosphatase treatment occurred. These reactions were analysed by agarose gel electrophoresis. Ligated products were then transformed into competent *E. coli* G1724 (sections 2.4.2 and 2.4.3), which allows inducible expression of the fusion protein at 30°C. (The alternative host strain G1698 was not required, this strain is used if the induction temperature is lower than 30°C, due to expression of insoluble recombinant protein at 30°C.) Figure 19 below shows a schematic representation of each of the recombinant plasmids after ligation.
Figure 19: Schematic maps of each recombinant plasmid. A - pTrxEBNA1, B - pTrx18VCA, C - pTrxEA, and D - pTrxBARF0. Plasmid sizes are indicated at the centre of each diagram.

Figure 20 shows DNA agarose gel analysis of each of the recombinant plasmids after ligation.
3.2.3 Restriction Analysis

Two restriction enzymes were used for the analysis of each plasmid, to verify that selected clones had the correct insert in the correct orientation. The BamHI digest indicated if the insert was of the correct size. The second enzyme chosen for each differentiated between clones containing the insert in the rightward orientation or the leftward orientation, due to differences in the fragment sizes. Table 6 below shows a list of the expected fragment sizes.
Table 6: Restriction Analysis, predicted fragment sizes from recombinant plasmids.

Differences in the banding pattern, which can be observed on an agarose gel are highlighted in bold type.

The patterns observed on agarose gel electrophoresis were as predicted for a number of clones, those showing the required pattern were chosen for growth and induction. Figures 21 to 23 show the restriction analysis of the four clones that were chosen for expression. The banding patterns were as predicted in table 6. In figure 21 a BamH1 digest shows the insert re-excised from the cloning vector, this digest showed that the insert was of the correct size. Further restriction analysis showed that the insert was in the correct orientation for expression of the fusion protein (figures 22 and 23).
Figure 21: DNA agarose gel analysis of BamHI digest of each recombinant plasmid. Lane 1 - DNA ladder, lane 2 - pTrxpl8, lane 3 - pTrxEBNA1, lane 4 - pTrxBARFO, lane 5 - pTrxEA

Figure 22: DNA agarose gel analysis of SmaI digest of each recombinant plasmid, on a 1.5% agarose gel. Lane 1 - DNA ladder, lanes 2-5 - SmaI digest of pTrxFus, pTrxEA, pTrxEBNA1 and pTrxBARFO respectively
3.3 Expression of Recombinant Protein

3.3.1 Growth and Induction

A number of clones containing the correct inserts were grown and induced to express each particular protein. Ten millilitre cultures of each were grown and induced as described in section 2.4.5. After harvesting of the cells, a crude sample of each was analysed by SDS-PAGE. This was done by resuspending the cell pellet from 0.5 ml of culture, 4 hours post-induction, in 50 μl sample buffer (appendix section III) and electrophoresis on a 10% SDS-PAGE gel.

Clones showing the highest levels of induction of recombinant protein were separated into soluble and insoluble fractions by repeated sonication and freeze-thaw cycles (section 2.4.5). It was found that all recombinant proteins were soluble when expressed at 30°C, therefore it was not necessary to use the G1698 E.coli strain for induction at lower temperatures. In summary, novel bands corresponding to the appropriate predicted molecular weights of each protein were found by SDS-PAGE.

Figure 23: DNA agarose gel analysis of Rsal digest of pTrx18, on a 2% agarose gel. Lane 1 - DNA ladder, lane 2 - pTrx18 cut with Rsal
Figure 24: Expression of soluble recombinant p18VCA in E.coli. Soluble and insoluble fractions of Trx and rVCAp18 after three rounds of sonication, freeze-thaw cycles. Lane 1 - Protein markers, lanes 2,3 - Trx soluble fraction, insoluble fraction, lanes 4,5 - rVCAp18 soluble fraction, insoluble fraction. Lanes 6,7 and 8,9 show soluble and insoluble fractions of two more rVCAp18 clones.

Three individual clones of rVCAp18 were analysed for soluble expression of the fusion protein. Figure 24 shows that upon induction at 30°C each expressed a soluble protein. Thioredoxin was used as positive control to show that the cell lysis was effective in separating the cell contents into a soluble and an insoluble fraction, and that thioredoxin itself was soluble when expression was induced at 30°C.
Figure 25: Soluble fraction of rBARFO, after three rounds of sonication and freeze-thaw.
Lane 1 - rBARFO soluble fraction, lane 2 - protein markers

Similar to rVCAp18, rBARFO was also found to be soluble when expression was induced at 30°C. As can be seen in figure 25, the level of expression for this clone was quite high. However, it was found subsequently that expression of the rBARFO fusion protein on a large scale (400 ml culture) was not possible. When plasmid DNA was analysed, it could be seen that the insert was still present, but the fusion protein was not expressed under the conditions used for each of the other fusions, on a larger scale. Expression was never recovered and BARFO clones were subsequently abandoned.

Variations on cell preparation were carried out by either omitting sonication or freeze-thaw, and/or varying the length of the sonication time. As described in section 2.4.5, the cells were sonicated for 3*10 s bursts, followed by rapid freeze-thaw, this was repeated twice. Figures 26 and 27 show the effect of increasing the sonication time, on the level of soluble fusion protein released from the cells, without freeze-thaw, for both Trx and rEBNA1, to investigate these parameters. For both rTrx and rEBNA1, the level of
soluble recombinant protein released gradually increased with increase in sonication time, reaching a peak at 150 s.

Figure 26: Effects of increasing sonication time without freeze-thaw, on Trx. Lanes 1-6 - 30 s, 60 s, 90 s, 120 s, 150 s, 180 s sonication, lane 7 - protein markers.

Figure 27: Effects of increasing sonication time without freeze-thaw, on rEBNA1. Lane 1 - Protein markers, lanes 2-5 - 30 s, 60 s, 90 s, 120 s, 150 s sonication.

It was found that the freeze-thaw cycles could be omitted without any loss in protein yield, and that the optimum sonication time was 150-180 s.
Figure 28 shows two clones of rEA expressed in the soluble form, released after 150 s sonication. The level of expression of this protein is considerably lower than that of each of the other recombinants.

Figure 28: Crude preparations and the soluble fraction after sonication of both Trx and rEA. The soluble fraction was isolated after 150 s sonication without freeze-thaw. Lane 1 - protein markers, lanes 2,3 - Trx crude sample, soluble fraction, lanes 4,5 and 6,7 - crude and soluble fraction samples of rEA clones, lanes 8,9 - rEA with the EA gene in the reverse orientation, crude and soluble fraction.

It can be seen in lanes 8 and 9 that when the BMRF1 ORF was cloned in the reverse orientation that no fusion protein was expressed.
3.3.2 Purification of Recombinant Proteins

Using the Thiofusion expression system, the fusion protein may be located at the adhesion zones (Lunn et al., 1982) (sites on the cytoplasmic side of the inner membrane which are osmotically sensitive, where native thioredoxin localises) In this case the protein could be purified by osmotic shock Alternatively, as thioredoxin is a heat stable protein (Holmgren, 1985), and this stability may be conferred to the fusion protein, this would allow purification by heat treatment at 80°C

3.3.2.1 Osmotic Shock

The rVCAp18 and rEA were tested for purification by osmotic shock (section 2.4.7) In brief, this involved incubation of the cells in a TE buffer with a high sucrose concentration, followed by incubation in a TE buffer without sucrose, thus causing the cells to burst.

![Figure 29: Purification of Trx and rEA by osmotic shock. Lane 1 - Trx shockate, lane 2 - Trx pellet, lane 3 - rEA shockate, lane 4 - rEA pellet, lane 5 - protein markers](image)

Figure 29: Purification of Trx and rEA by osmotic shock. Lane 1 - Trx shockate, lane 2 - Trx pellet, lane 3 - rEA shockate, lane 4 - rEA pellet, lane 5 - protein markers
Figure 30: Purification of rVCAp18 by osmotic shock. Lane 1 - rVCAp18 soluble fraction after somcation, lane 2 - rVCAp18 pellet after osmotic shock, lane 3 - rVCAp18 shockate, lane 4 - protein markers

Figure 29 shows Trx released into the shockate after osmotic shock, however both rEA (lane 4, figure 29), and rVCAp18 (lane 2, figure 30) remained in the pellet fraction. The result was similar for all other recombinants, with the recombinant protein remaining in the pellet fraction. Unlike native thioredoxin, it would appear that the fusion proteins are not localised at the adhesion zones. Therefore, osmotic shock could not be used as a method of purification of the thioredoxin fusions.

3.3.2.2 Heat Treatment

The rVCAp18 was also chosen for purification by heat treatment (section 2.4.8) as this clone showed the highest level of expression of recombinant protein. In summary, this method involved heating the soluble fraction of the cell lysate to 80°C. Samples were removed over a time course of 10 min, each sample was centrifuged to separate the precipitated proteins and both fractions were analysed by SDS-PAGE. Most proteins precipitate after 1 min at 80°C, while some thioredoxin fusions are stable for 10 min at this temperature (Holmgren, 1985)
Thioredoxin could be seen to remain in the soluble fraction after incubation at 80°C for up to 10 min (Figure 31, lanes 4, 6, 8 and 10). There was some loss to the pellet fraction after 5 min (Figure 31, lane 7). Heat treatment of rVCAp18 showed that after incubation at 80°C for 10 min some of the fusion protein was separated from the remainder of the cellular protein, however a substantial amount of the recombinant protein is precipitated also (Figure 31, lanes 18 and 19). At shorter incubation times of 5-8 min there is also a high quantity of the recombinant protein in the pellet (Figure 32, lanes 8-10, pellet) and some of the native cellular proteins remaining in the supernatant (Figure 32, lanes 12-14, supernatant).
Figure 32: Purification of Trx and rVCAp18 by heat treatment at 80 °C. Lane 1 - Protein markers, lane 2 - crude Trx, lanes 3,4 - 5 min, 8 min HT pellet, lanes 5,6 - 5 min , 8 min HT supernatant Lane 7 - crude rVCAp18, lanes 8 - 11, - 5, 7, 8, 10 min HT rVCAp18 pellet, lanes 12-15 - 5, 7, 8, 10 min HT rVCAp18 supernatant, lane 16 - protein markers

A 10 min incubation at 80°C was required to remove the majority of native *E. coli* proteins resulting in substantial loss of recombinant protein. Heat treatment was not used to purify the other recombinant proteins, the level of purity obtained was not considered sufficient to compensate for the level of recombinant protein lost.

3.3.2.3 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation is used extensively as the first stage in protein purification. This method involves precipitation of particular proteins using varying concentrations of ammonium sulphate (AS). In this case, the first stage involves the precipitation of the majority of the cellular proteins without precipitating recombinant protein. The highest percentage AS at which the recombinant protein remains soluble is first determined. This soluble fraction is then used for further precipitation at higher concentrations of AS. During this stage the recombinant protein is precipitated from
the remaining native cellular proteins. Large-scale, 400 ml cultures were grown and induced for purification by this method.

Figure 33: Ammonium sulphate (AS) precipitation of rVCAp18 - 1st cut. Lane 1 - Protein markers, lanes 2,3 - supernatant (S), pellet (P) 20% AS, lanes 4,5 - S,P 25% AS, lanes 6,7 - S,P 28% AS, lanes 8,9 - S,P 30% AS, lanes 10,11 32% AS, lane 12 - S 35% AS, lane 13 protein markers.

Figure 34: Ammonium Sulphate precipitation of rEBNA1 - 1st and 2nd cut. Lane 1 Protein markers, lane 2 - crude sample rEBNA1, lanes 3 - supernatant 35% AS, 1st cut, lanes 4,5 - supernatant, pellet 48% AS, 2nd cut.
At 28% AS it can be seen that rVCAp18 was completely precipitated (Figure 33 lane 7). As there was a very low level of native proteins in this fraction, it was decided not to proceed with a second ammonium sulphate cut, and to use resolubilised pellet after the 28% AS cut for further analysis of the recombinant p18VCA Figure 34 shows the products of two AS cuts with rEBNA1, after the first cut with 35% AS, rEBNA1 was in the soluble fraction (Figure 34, lane 3), a considerable amount of native proteins were precipitated. After the second cut at 48% AS, rEBNA1 was precipitated (lane 5), some E.coli proteins remained rEA was also purified by AS (not shown), this fusion protein was precipitated at 35% AS. After AS precipitation there was still a substantial amount of contaminating protein in each sample, which could cause non-specific signals in Western Blotting or ELISA analysis of sera. Many human sera are likely to contain antibodies to E.coli antigens, therefore it was important to minimise the level of native protein remaining in each protein preparation.

### 3.3.2.4 Phenylarsine Oxide (PAO) Agarose

In order to further reduce the level of background proteins an alternative method of purification was chosen. A specifically activated resin was used, this resin is an agarose-based support covalently modified with phenylarsine oxide (PAO) (Sigma catalogue 1998), which is especially designed for the affinity purification of proteins containing vicinal dithiols, this includes thioredoxin and thioredoxin fusions. These proteins reversibly bind the phenylarsine oxide in the ‘Thiobond’ resin through these dithiols. Other proteins are washed away, and the purified thioredoxin fusion protein is eluted using β-mercaptoethanol (β-mer). The conditions for purification were set according to those required for the purification of thioredoxin using this method, this involved considerable optimisation and variation from the manufacturers instructions (section 2.4.11). The concentration of β-mer used for column equilibration, washing and elution had to be altered. A number of variations of β-mer concentration in the buffers for each stage were carried out (data not shown). Ultimately all of the recombinant proteins were purified by this method, using the same conditions. The column was equilibrated with 3mM β-mer, washed with 0.25 mM β-mer and fusion proteins were eluted with 500mM β-mer.
Figure 35: Purification of Trx using PAO agarose. Lane 1 - Protein markers, Lane 2 - Trx soluble fraction after sonication, lane 3 - flow through, lanes 4,5 - wash with 0.25 mM β-mer, lane 6,7 elution with 250 mM β-mer, lanes 8,9 elution with 500 mM β-mer

Although the recombinant was released with 250 mM β-mer (figure 35, lanes 6 and 7), there was some protein remaining which was subsequently released using 500 mM β-mer (figure 35, lanes 8 and 9). There was no increase in the background proteins, therefore it was suitable to use 500 mM β-mer for elution as just one wash was required for complete elution.
Figure 36: Purification of rEBNA1 using PAO agarose. Lane 1 - Protein markers, lane 2- rEBNA1 soluble fraction after sonication, lane 3 - flow through, lanes 4,5 - wash with 0.25 mM β-mer, lane 6,7 elution with 500 mM β-mer

As can be seen in figure 36 lane 6, there are two native E.coli proteins which are also eluted, at ~20 kD and at ~100 kD. There does not appear to be any native thioredoxin present.

Figure 37: Purification of rVCAp18 and rEA using PAO agarose. Lane 1 - rVCAp18 soluble fraction after sonication, lane 2 - flow through, lanes 3,4 wash with 0.25 mM β-mer, lanes 5,6 - elution with 500 mM β-mer Lane 7 - rEA soluble fraction after sonication, lane 8 - flow through, lanes 9,10 - wash with 0.25 mM β-mer, lanes 11,12 - elution with 500 mM β-mer.
It can be seen that the flow through and the washes for both rVCAp18 and rEA contain recombinant protein (Figure 37 lanes 2, 3, 8 and 9). This may be due to the agarose binding capacity being reached, the columns could be reused up to 4 times but with each use the agarose binding capacity decreased (not shown). Figures 35 - 37 show that each of the proteins appears to have been purified to a greater extent than by ammonium sulphate precipitation, i.e. a reduction in the degree of background protein present. In this way the background reactivity with human serum can be minimised. This level of purification is likely to be required if the recombinant antigens are to be used effectively as diagnostic reagents. However, it was not possible to completely remove all *E. coli* proteins even when using a thioredoxin selective purification method.

### 3.3.2.5 Protein Assay and Densitometry

After purification each protein preparation was dialysed exhaustively against PBS pH 7.4, to remove the remaining β-mer. These samples were assayed by the BCA assay as described in the manufacturers instructions (PIERCE product no 23225). As there were other cellular proteins still present, densitometry was carried out in order to quantify the amount of recombinant protein in each preparation. This involved running a sample of each on SDS-PAGE beside a set of standards of known concentration. The SDS-PAGE gels used for densitometry are shown in figures 38 and 39.
Figure 38: SDS-PAGE gel analysis of protein standards and rVCAp18 and rEBNA1 (purified using PAO agarose) for densitometry. Lanes 1-6 - BSA-NIP-1, 2, 3, 4, 5 and 6 μg respectively, lane 7 - rVCAp18 uninduced, lanes 8,9 - rVCAp18 induced, lane 10 - rEBNA1

Figure 39: SDS-PAGE gel analysis of protein standards and rEBNA1 and rEA (purified using PAO agarose) for densitometry. Lanes 1-6 - BSA-NIP-1, 2, 3, 4, 5 and 6 μg respectively, lane 7 - rEBNA1 uninduced, lane 8 - rEBNA1 induced, lanes 10,11 - rEA induced.

To use dilutions of these samples may have proven more accurate, however as the BCA assay carried out on each of the recombinants showed the level of total protein concentration to fall within the level of protein standards used the results the neat
samples were used for densitometry. It can be seen in lane 7, figure 38, and in lane 7 figure 39, that there is a low level of fusion protein present in the uninduced samples of rVCAp18 and rEBNA1, this was also the case for rEA. This leaky expression was not apparent before purification by PAO agarose as the level of E.coli proteins present masked the fusion protein when analysed on SDS-PAGE. These uninduced samples could not be used as negative controls for ELISA analysis, it was therefore necessary to prepare a negative control sample from G1724 cells without any plasmid DNA (Trx'+G1724), thus eliminating any possible expression of recombinant protein. The Trx'+G1724 cellular proteins were purified by PAO agarose under the same conditions also.

A BCA assay was carried out to determine the total protein concentration, the densitometry reading for each recombinant was compared to that of each of the protein standards, and the concentration was determined using this ratio and the protein assay results. Table 7A shows the concentration of each protein and the total protein concentration of each purified sample.

<table>
<thead>
<tr>
<th>rProtein</th>
<th>rProtein conc (μg/ml)</th>
<th>Total Protein conc (μg/ml)</th>
<th>Native E.coli protein conc (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rEBNA1</td>
<td>215</td>
<td>362</td>
<td>147</td>
</tr>
<tr>
<td>rVCAp18</td>
<td>178</td>
<td>328</td>
<td>150</td>
</tr>
<tr>
<td>rEA</td>
<td>420</td>
<td>628</td>
<td>208</td>
</tr>
</tbody>
</table>

Table 7A: Protein Concentration of each Recombinant Protein, as determined by the BCA assay and densitometry, (rProtein - recombinant protein).
Table 7B: Approximate concentration of each of the recombinant proteins, after affinity purification using PAO agarose.

The yield of total protein (Table 7A, column 3) represents the total protein remaining after purification using PAO agarose.

Western blotting analysis using pre-characterised sera was used to confirm the antigenicity of three recombinant antigens - rVCAp18, rEBNA1, rEA (described below in section 3.4.2)

3.4 Antigenicity of rProteins

3.4.1 Serum Characterisation

Human antisera were donated from a number of different sources (see section 2.1.2) for analysis using the recombinant antigens. Each of these serum samples had previously been characterised (prior to receiving the serum samples) for anti-EBV antibodies using a commercially available kit, as listed in section 3.1. The sera from randomly chosen normal individuals had not previously been characterised with respect to anti-EBV antibodies.

3.4.1.1 Infectious Mononucleosis Sera

The IM+ sera were tested using an anti-EBV capsid IgM indirect immunofluorescence assay (IFA). The sera are pretreated with GullSORB to remove IgG antibodies thus preventing IgG/rheumatoid factor complexes from forming and preventing interference...
from IgG during the test procedure. The assay is carried out using Gull slides coated with EBV capsid antigen gp125, purified from the EBV-positive P3HR-1 cell line.

3.4.1.2 Viral Capsid Antigen (VCA) -Positive and -Negative sera

These sera were tested using the Gull EB VCA IgM ELISA (Gull product no. EBE150). In this assay, purified EBV VCA gp125 from the EBV-positive P3HR-1 cell line, is used to coat a 96-well plate. The serum is pre-treated with a PBS-based diluent containing goat anti-human IgG. This absorbent prevents IgG/rheumatoid factor complexes from forming and prevents interfering competition from IgG during the test procedure. (Rheumatoid factor is an IgM type antibody which reacts with IgG antibodies to form immune complexes.) The conjugate used in this test kit is anti-human IgM alkaline phosphatase. A negative control serum, positive control serum, and a reference serum are included to aid interpretation of the ELISA results. The reference serum is minimally reactive for this kit, this control sample is set up in triplicate for each test run. Optical Density (OD) readings for test samples which are greater than the mean OD of the 3 reference samples, are considered positive.

3.4.1.3 EBNA -Positive and -Negative Sera

The Biotest anti-EBV Recombinant EBNA IgG ELISA was used for characterisation of these sera. A recombinant EBNA1 (46 kD), consisting of the C-terminal part of the native EBNA1 (47.2%), is used to coat the ELISA plate. A goat anti-human IgG peroxidase conjugate is used for detection. Specimens are considered positive in this test when the OD is greater than the mean of the negative controls plus a factor of 0.150.

3.4.1.4 EBV-Positive Sera and CMV-Positive Sera

The EBV-positive sera were characterised using the Baxter Bartels EBV IgM EIA. The antigen used in this assay is EBV VCA gp125 purified from P3HR-1 cells. Patient serum is diluted with a specimen diluent which contains an absorbent to remove IgG, thus preventing IgG/rheumatoid factor complexes forming and also preventing interfering competition from IgG during the test procedure. The conjugate used is anti-human IgM.
alkaline phosphatase, and both positive and negative control specimens were included. The CMV-positive sera were characterised using the Baxter Bartels CMV IgM EIA. This kit consisted of EIA wells coated with purified CMV antigen (AD strain). As for the EBV IgM EIA the specimen diluent contained an absorbent for the removal of IgG. An anti-human alkaline phosphatase conjugate is also used for detection.

3.4.1.5 Heterophile Antibody Test

A number of the IM-positive sera, normal sera and VCA-positive sera were selected for testing using the Organon Teknika, Monosticon DRI-DOT Infectious Mononucleosis diagnostic kit. This is a 2-minute disposable hemagglutination slide test for the qualitative determination of IM antibody. This test is based on the Paul-Bunnell heterophile antibody test as described in section 15.2. In addition to EBV-specific antibodies, the early phase of IM is characterised by a general increase in total IgM, IgG and IgA, which is consistent with virus-driven polyclonal activation of the B cell system. The presence of heterophile antibodies in sera of IM patients was originally detected by agglutination of sheep, horse or bovine blood cells presenting heterophile antigens on their surfaces. Heterophile antibodies are usually detected less than one week after onset of IM, peak at two-five weeks and can be detected, at low levels, a year after onset of illness (Niederman et al., 1968; Evans et al., 1975). A combination of horse erythrocytes and sheep erythrocytes are used and guinea pig antigen is included to neutralise Forssman antibody (Forssman antibody is a heterophile antibody that reacts with Forssman antigen, a heterophile antigen, which occurs in a wide variety of unrelated animals), which may influence the result. Horse erythrocytes have been shown to be more sensitive than sheep erythrocytes for detection of IM antibody (Lee et al., 1968). This test was carried out by the author, the serum and test reagent were incubated at room temperature for 2 min with gentle rocking (as per instructions). Agglutination indicates a positive result, lack of agglutination indicates a negative result. The level of agglutination detected is relative to the amount of heterophile antibody present. This test may be used quantitatively also. See figure 40 and table 8 for the results obtained with the sera tested.
<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Type</th>
<th>Result</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>7127/97</td>
<td>IM</td>
<td>+</td>
<td>no agglutination</td>
</tr>
<tr>
<td>9217/97</td>
<td>IM</td>
<td>++</td>
<td>slight agglutination</td>
</tr>
<tr>
<td>12847/97</td>
<td>IM</td>
<td>+++</td>
<td>strong agglutination</td>
</tr>
<tr>
<td>6370/98</td>
<td>IM</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>6925/98</td>
<td>IM</td>
<td>+</td>
<td>very slight agglutination</td>
</tr>
<tr>
<td>7083/98</td>
<td>IM</td>
<td>+</td>
<td>very slight agglutination</td>
</tr>
<tr>
<td>12755/98</td>
<td>IM</td>
<td>+</td>
<td>very slight agglutination</td>
</tr>
<tr>
<td>15941/98</td>
<td>IM</td>
<td>-</td>
<td>agglutination</td>
</tr>
<tr>
<td>9244/98</td>
<td>IM</td>
<td>++</td>
<td>slight agglutination</td>
</tr>
<tr>
<td>11531/98</td>
<td>IM</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>12707/98</td>
<td>IM</td>
<td>+++</td>
<td>strong agglutination</td>
</tr>
<tr>
<td>14769/98</td>
<td>IM</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>15038/98</td>
<td>IM</td>
<td>+++</td>
<td>strong agglutination</td>
</tr>
<tr>
<td>15386/98</td>
<td>IM</td>
<td>+</td>
<td>very slight agglutination</td>
</tr>
<tr>
<td>7435</td>
<td>Normal</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>7436</td>
<td>Normal</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>7438</td>
<td>Normal</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>7439</td>
<td>Normal</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>7442</td>
<td>Normal</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>7443</td>
<td>Normal</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>8413</td>
<td>VCA</td>
<td>-</td>
<td>no agglutination</td>
</tr>
<tr>
<td>8415</td>
<td>VCA</td>
<td>++</td>
<td>slight agglutination</td>
</tr>
<tr>
<td>7611</td>
<td>VCA</td>
<td>+++</td>
<td>strong agglutination</td>
</tr>
</tbody>
</table>

Table 8: Monosticon DRI-DOT results from a selection of sera.
Figure 40: Photograph of the Monosticon DRI-DOT test cards after serum was added. 1- No agglutination with serum no 8413, 2 - Slight agglutination with serum no 8415, 3 - Strong agglutination with serum no 7611 Each of these sera were characterised as VCA-positive using the Gull EB VCA IgM ELISA
These results show inconsistencies between the IM sera tested using the Gull anti-VCA IgM IFA test and the Monosticon test. Only 9 of 14 IM-positive sera give a positive result using this method. The level of agglutination was also variable, ranging from immediate strong agglutination to very slight agglutination after 2 min. All the normal sera were negative as expected and 2 of 3 VCA-positive sera were positive. Some VCA-positive sera would be expected to give a positive result using this test, also as the presence of anti-VCA IgM antibodies is usually indicative of infectious mononucleosis.

3.4.2 Western Blot Analysis with IM Sera and Normal Sera

3.4.2.1 rVCAp18

Prior to purification of the recombinant proteins with PAO agarose, western blot analysis was carried out for rVCAp18, using the soluble fraction after cell lysis. This was done to ensure immunoreactivity of the recombinant antigen before more complete purification of the protein on a larger scale. A number of IM-positive sera were chosen for this analysis. The procedure was as described in section 2.4.10. An anti-human Ig alkaline phosphatase conjugate was used for detection. Each of the sera could be seen to have some degree of reactivity to the recombinant rVCAp18 (See figure 41). There was no antibody response to Thioredoxin expressed from pTrxFus, this showed that the response to the fusion protein was specific for the p18VCA moiety. Therefore, it was not considered necessary to cleave the thioredoxin moiety from the recombinant fusion proteins using enterokinase. The level of non-specific anti-E.coli antibodies was different for each serum sample (see higher molecular weight bands in figure 41, lanes 9, 10 and 11). The degree of background reactivity was considerably lower than the signal found with rVCAp18. However, in an alternative assay such as ELISA or DOT blot this background signal could result in false-positive interpretation of results.
Figure 41: Western blot analysis of Trx and rVCAp18 after sonication, with six IM-positive sera. Lane 1 - protein markers, lanes 2,3 - Trx, rVCAp18 probed with serum 962, lanes 4,5 - Trx, rVCAp18 probed with serum 10211, lanes 6,7 - Trx, rVCAp18 probed with serum 9217, lanes 8,9 - rVCAp18, Trx probed with serum 7217, lanes 10,11 - rVCAp18, Trx probed with serum 7126, lanes 12,13 - Trx, rVCAp18 probed with serum 12874 (10 μl of each protein preparation was loaded, the sera were diluted 1/100 in BLOTTO)

Those found to give the strongest signal were further analysed in a repeat experiment. The protein preparation was diluted in the range 1:2 to 1:64 in PBS to find the limiting dilution for detection using serum diluted 1/100. As can be seen in figures 42 and 43 the limiting dilution ranged between 1:2 and 1:32 for two particular sera.
Figure 42: Detection of rVCAp18 by western blotting, with IM-positive serum 10211. Lane 1 - Protein markers, lanes 2-8 - rVCAp18 neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64.

Figure 43: Detection of rVCAp18 by western blotting, with IM-positive serum 7126. Lane 1 - Protein markers, lanes 2-8 - rVCAp18 neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64.

3.4.2.2 Blotting of all Proteins after Purification using PAO agarose

The three recombinant antigens were combined in equal concentrations, using the protein concentrations determined by BCA assay and densitometry (1 μg of each rProtein was loaded per lane). The sample was run on a 10% SDS-PAGE gel for western blotting with a number of IM-positive sera and Normal sera. In this case two secondary
antibodies were used, an anti-human IgM peroxidase conjugate, and an anti-human IgG peroxidase conjugate. As discussed earlier, it is important to establish whether the antibody reaction is IgG or IgM, in order to determine the type of infection or the stage of disease. Table 9 below shows the expected IgG and IgM Ab responses to each of the recombinant antigens of normal (EBV+) and IM+ sera.

<table>
<thead>
<tr>
<th>Serum type</th>
<th>IgM VCA</th>
<th>IgG VCA</th>
<th>IgM EBNA</th>
<th>IgG EBNA</th>
<th>IgG EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (EBV+)</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>IM+</td>
<td>+</td>
<td>++</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 9: Predicted IgG and IgM Ab responses of normal (EBV+) and IM+ sera. [+ denotes positive; - denotes negative; ++ denotes elevated positive]

Figure 44: Detection of IgG Ab response to the 3 recombinant EBV antigens. Lanes 1-10 were probed with 10 different IM-positive sera (7083, 10035, 10188, 12344, 14769, 12755, 9244, 16874, 14495, 12344) lanes 11-20 were probed with 10 different normal sera (7458, 7459, 7460, 7461, 7462, 7463, 7464, 7465, 7466, 7467) lane 21 - protein markers.
As can be seen in figure 44, almost all the normal sera (except 7458 and 7462) showed an IgG response to rVCAp18, and a small number showed slight reactivity with rEBNA1 (7459 and 7463) and rEA (7459 and 7461). The IM+ sera showed no response to rEBNA1 or rEA, and only a few sera were seen to be reactive with rVCAp18 (10188, 16874, 14495).

Figure 45: Detection of IgM Ab response to the 3 recombinant EBV antigens. Lane 1 - protein markers, lanes 2-11 were probed with 10 different IM-positive sera (7083, 10035, 10188, 12344, 14769, 12755, 16874, 9244, 14495, 12344) lanes 12-21 were probed with 10 different normal sera (7458, 7459, 7460, 7461, 7462, 7463, 7464, 7465, 7466, 7467).

Figure 45 shows the strong IgM response of the IM+ sera to rVCAp18 in the majority of the sera tested (12344 shows a weak response, lanes 5 and 11, 10188 shows no response, lane 4). Three IM+ sera showed an IgM response to rEA (7083, 10035 and 9244, lanes 2,3 and 9 respectively), and as expected there was no IgM response to rEBNA1 with any IM+ serum. There was no IgM responses to any of the recombinant proteins for the normal sera with the exception of 7465, lane 19, which showed an anti-rVCAp18 IgM response. IgM+ VCA, IgM- EBNA1 is the typical response for patients with IM as described earlier (see able 19). IgM- VCA, IgM- EBNA1 is the typical response for sera from EBV+ individuals without IM. All the normal sera tested appear to be EBV+ as determined by the detection of IgG antibody response to rVCAp18 by western blot (shown in figure 44).
3.4.3 ELISA using Recombinant EBV Antigens

ELISA analysis of all sera was carried out as described in section 2.4.12, the procedure was adapted from a number of protocols and optimised for use with these recombinant EBV antigens and human serum (Crowther 1995). The wells were coated with 10 μg/ml of recombinant protein, and an equivalent amount of background protein was loaded for each antigen. This was determined using the concentration values obtained by BCA assay and densitometry. The *E. coli* G1724 strain, without plasmid, was grown and induced in an identical manner to that of each of the recombinants. The cells were harvested and purification was carried out using the PAO agarose also. In this way the background proteins expressed in each of the recombinants, which are not removed during purification, would be present and could be used to control for background in ELISA assays. As the concentration of background proteins varies for each recombinant, it was necessary to add the required amount of G1724 extract to each sample to make the concentration uniform throughout. An equivalent concentration of G1724 was used to coat wells as a negative control, and the total protein concentration was increased by 10 μg/ml (the concentration of each rProtein) using BSA. Table 10 below shows the volumes of each protein required per ml of sample preparation, to give a recProtein concentration of 10 μg/ml and a background protein concentration of 8.4 μg/ml (the rVCAp18 extract had the highest background protein concentration of 8.4 μg/ml, therefore the background protein concentration of each sample was increased to this level). Table 11 shows the assays carried out with each of the recombinant antigens and the types of sera tested.

<table>
<thead>
<tr>
<th>Protein type</th>
<th>recProtein conc. (μg/ml)</th>
<th>Volume of purified protein (μl/ml)</th>
<th>Volume of G1724 extract (μl/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1724</td>
<td>347</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>rEBNA1</td>
<td>215</td>
<td>46.5</td>
<td>3.8</td>
</tr>
<tr>
<td>rVCAp18</td>
<td>178</td>
<td>56</td>
<td>-</td>
</tr>
<tr>
<td>rEA</td>
<td>420</td>
<td>24</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 10: Details of sample preparation for ELISA assay.
<table>
<thead>
<tr>
<th>Serum type</th>
<th>rEBNA</th>
<th>rVCAp18</th>
<th>rEA</th>
<th>Anti-IgG</th>
<th>Anti-IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Normal</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VCA +/-</td>
<td>nd</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>EBNA +/-</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>EBV +</td>
<td>nd</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>CMV +</td>
<td>nd</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 11: The ELISA assays which were carried out. [(+) - indicates that the antigen was tested, and the antibody type tested for, IgG/IgM, (nd) - not done]

The expected serological profiles of normal (EBV+) and IM+ sera were specifically outlined in table 9. In general, IM+ sera would be expected to be anti-p18VCA IgG and IgM-positive, anti-EBNA1 IgG and IgM-negative and anti-EA-D IgG-positive. Normal sera may either be EBV-positive or -negative, however as 90% of the population are EBV-positive the expected profile would be as follows - anti-p18VCA IgG-positive, IgM-negative, anti-EBNA1 IgG-positive, IgM-negative and anti-EA-D IgG-positive/negative. Figures A - J show the results obtained from the ELISAs carried out with each of the recombinant antigens as detailed in table 11. The values used for graphing are averages of triplicate OD readings at 414 nm.
In figure A the anti-rVCAp18 IgM response is shown for four serum types. The IgM response is the first to develop at the early stages of IM, and peaks within the first 1-2 weeks (see figure 6), after which the level of IgM antibodies drops to zero after approximately 4 weeks. All but a few IM+ sera show an elevated level of anti-VCA IgM antibodies, all the normal sera show a low level of IgM antibodies to the recombinant p18VCA. These results correlate favorably with the results obtained by western blot
(section 3.4.2.2), none of the normal sera tested by western blot had shown the presence of IgM antibodies to the recombinant antigens (figure 45, lanes 12-21) The majority of the IM+ sera showed a strong signal by western blot (figure 45, lanes 2-11) and by ELISA However, one serum (12344) had a very low western blot signal, but the response by ELISA was high, OD @ 414nm = 0.616 Other sera with OD readings of approximately 0.6 had shown a stronger signal by western blot (figure 45, lanes 3 and 6) As expected the VCA+ sera gave a high IgM response, while the VCA- showed a low IgM response It is likely, although it cannot be confirmed (information not available), that the VCA+ sera were taken from patients with an IM first infection, as there is an IgM response to p18VCA

The anti-rVCAp18 IgG responses of both normal and IM+ sera can be seen in figure B The IgG response of the IM+ sera was lower than expected, the anti-p18VCA IgG antibody is normally elevated in IM+ patients However, as can be seen in figure 6 (section 1.5.1) it may take up to 4 weeks for the IgG titer to reach a peak If these serum samples were taken when the first onset of symptoms had shown then it would be expected that the IgG titer to p18VCA would be positive but still at a low level The majority of normal sera had a low level, but positive IgG response, this indicates that these normal sera were most likely EBV+ The IgM ELISA results and the IgG ELISA results correlate well with the western blot results for the normal and IM+ sera (section 3.4.2.2) However, 1 IM+ sera (10188) shows a very low ELISA result (OD @ 414nm = 0.058), while the western blot signal is quite strong (figure 44, lane 3)
Figure C shows the IgG responses to rEBNA. In general, both the EBNA+ and normal sera showed a higher anti-EBNA IgG response than that of the EBNA- and IM+ sera. Some of the normal sera showed a very low response comparable to EBNA- and IM+ sera. Two of 3 normal sera with a low anti-EBNA response also had very low anti-VCA
IgG and IgM responses, this may indicate that these samples were taken from patients who were EBV-negative. The other sample showed an elevated anti-VCA IgG response and a low anti-VCA IgM response, this patient may be in the final stages of IM (this cannot be confirmed as the health status of these patients is not known). The anti-rEBNA IgG response of the IM+ sera was low, as expected, with OD readings comparable to those of the EBNA- sera. All the sera tested by western blot showed antibody responses which correlated well with the ELISA results.

The anti-EBNA1 IgM responses of both the normal and IM+ sera were very low as might be expected, there were only 2 IM+ sera which gave a higher OD reading (figure D). In comparison with the anti-p18VCA IgM readings, the absolute OD values are comparable to the VCA- sera with the lowest level of response. Two IM+ sera (16874 and 14495) showed a high OD reading by ELISA (0.606 and 0.547 respectively), however there was no signal for either by western blot (figure 45, lanes 8 and 10 respectively).

Figure E

Anti-EA IgG Responses of Normal Sera and IM+ Sera

![Graph showing anti-EA IgG responses]

- Serum sample
The trend of the anti-EA IgG responses for normal and IM+ sera (figure E) are very similar, there is a substantial overlap in the responses for both serotypes. The normal sera may either have a positive or negative response while the IM+ sera would be expected to have a positive IgG response to EA. However, as for the anti-p18VCA IgG response it takes up to 3-4 weeks for the anti-EA IgG response to reach a peak before decreasing again to zero at about 5-6 weeks. If these samples were taken within the first or second week after the onset of symptoms one would expect these low level anti-EA IgG responses.

The anti-EA IgM responses were generally very low for both IM+ sera and normal sera, which is expected in most cases (figure F). A number of the IM+ sera gave a higher response to that of the normal sera, however, in comparison with the level of the IgM response to p18VCA these values were very low. Generally, it is the IgG response to EA which is of significance in diagnosis of IM, while the anti-EA IgA response has been shown to be of significance in the diagnosis of NPC (Henle and Henle, 1976, Nadala et al, 1996). The western blot results and ELISA results for all sera compare favorably for both IgG and IgM responses.
Figure G shows the general profile of IgG responses of the normal sera tested by ELISA, to each of the recombinant antigens. As stated earlier, normal sera may have a positive IgG response to each of these antigens, if they are EBV+. In the cases of patients who are EBV-negative, there would be a negative response, however, these cases are relatively rare. The majority of the normal sera tested show a low level IgG response to each of the antigens, which may be due to the background proteins present in each preparation, as these recombinant proteins were not purified to homogeneity.
The IgM profile of the normal sera (figure H) was also as might be predicted, in general the level of IgM antibodies in serum of patients without an active infection is very low.

Figure I shows the IgG profile of IM+ sera tested by ELISA with each recombinant antigen. The mean OD values for anti-p18VCA and anti-EA IgG were higher than that of the anti-EBNA IgG. In general the IgG responses are low, due to the fact that these
samples were probably taken at early onset of symptoms before the anti-p18VCA and anti-EA IgG responses have elevated

Figure J

The IgM profile for the IM+ sera was as might be expected, IM+ sera are anti-p18VCA IgM-positive, anti-EBNA IgM-negative and anti-EA IgM-negative. This trend can clearly be seen in figure J. There was a low level response to EBNA and EA, it is possible that this may be due to the background proteins present in each protein preparation.

3.4.4 Establishment of an ELISA Reference Value

The western blot results from section 3.4.2.2 were used for comparison with ELISA results in order to establish a baseline OD value. An OD value above this reference point would indicate a positive result, and any OD reading below would be considered negative. Ten normal sera and 9 IM positive sera were compared for both IgG responses and IgM responses. The following table shows ELISA vs Western blot results for each of the chosen sera.
<table>
<thead>
<tr>
<th>Serum</th>
<th>rVCap18 ELISA IgG</th>
<th>rVCap18 WB IgG</th>
<th>rEBNA1 ELISA IgG</th>
<th>rEBNA1 WB IgG</th>
<th>rEA ELISA IgG</th>
<th>rEA WB IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7083</td>
<td>0.334</td>
<td>+</td>
<td>0.292</td>
<td>-</td>
<td>0.279</td>
<td>+</td>
</tr>
<tr>
<td>10035</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0.137</td>
<td>+</td>
</tr>
<tr>
<td>10188</td>
<td>0.058</td>
<td>++</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>12344</td>
<td>0.127</td>
<td>+</td>
<td>0.043</td>
<td>-</td>
<td>0.092</td>
<td>-</td>
</tr>
<tr>
<td>14769</td>
<td>0.124</td>
<td>+</td>
<td>0</td>
<td>-</td>
<td>0.089</td>
<td>-</td>
</tr>
<tr>
<td>12755</td>
<td>0.122</td>
<td>-</td>
<td>0.039</td>
<td>-</td>
<td>0.107</td>
<td>-</td>
</tr>
<tr>
<td>16874</td>
<td>0.095</td>
<td>-</td>
<td>0.052</td>
<td>-</td>
<td>0.070</td>
<td>+</td>
</tr>
<tr>
<td>9244</td>
<td>1.516</td>
<td>+++</td>
<td>0.158</td>
<td>-</td>
<td>0.157</td>
<td>-</td>
</tr>
<tr>
<td>14495</td>
<td>0.621</td>
<td>++</td>
<td>0.179</td>
<td>-</td>
<td>0.486</td>
<td>+</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7458</td>
<td>0.110</td>
<td>+</td>
<td>0.186</td>
<td>-</td>
<td>0.119</td>
<td>+</td>
</tr>
<tr>
<td>7459</td>
<td>0.287</td>
<td>++</td>
<td>0.273</td>
<td>+</td>
<td>0.057</td>
<td>+</td>
</tr>
<tr>
<td>7460</td>
<td>0.748</td>
<td>+++</td>
<td>0.171</td>
<td>-</td>
<td>0.070</td>
<td>-</td>
</tr>
<tr>
<td>7461</td>
<td>0.262</td>
<td>++</td>
<td>0.302</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>7462</td>
<td>0</td>
<td>+</td>
<td>0.146</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>7463</td>
<td>0.216</td>
<td>++</td>
<td>0.498</td>
<td>++</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>7464</td>
<td>0.315</td>
<td>++</td>
<td>0.268</td>
<td>+</td>
<td>0.018</td>
<td>+</td>
</tr>
<tr>
<td>7465</td>
<td>0.213</td>
<td>+++</td>
<td>0.244</td>
<td>+</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>7466</td>
<td>0.224</td>
<td>++</td>
<td>0.237</td>
<td>+</td>
<td>0.015</td>
<td>-</td>
</tr>
<tr>
<td>7467</td>
<td>0.453</td>
<td>++</td>
<td>0.539</td>
<td>+</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 12: ELISA vs Western Blot anti-IgG results of 9 IM+ sera and 10 normal sera. WB - western blot. The number of (+) denotes the level of intensity of the western blot signal. The values in bold denote the highest ELISA value for which the corresponding WB result was negative and the next highest ELISA result for which the corresponding WB result was positive, for each recombinant protein.

For rVCap18 any OD reading greater than 0.124 (serum 14769) was a positive result, for rEBNA1 an OD reading greater than 0.302 (serum 7461) indicates a positive result and for rEA an OD reading above 0.279 (serum 7083) was positive. Combining these data a minimum value can be obtained above which is designated a positive result for any of the three recombinant proteins. An OD reading @ 414nm greater than or equal to 0.302 would indicate a positive IgG reaction to the particular protein being assayed.
<table>
<thead>
<tr>
<th>Serum</th>
<th>rVCApl8 ELISA IgM</th>
<th>rVCApl8 WB IgM</th>
<th>rEBNA1 ELISA IgM</th>
<th>rEBNA1 WB IgM</th>
<th>rEA ELISA IgM</th>
<th>rEA WB IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7083</td>
<td>1.148</td>
<td>+++</td>
<td>0.101</td>
<td>-</td>
<td>0.062</td>
<td>++</td>
</tr>
<tr>
<td>10035</td>
<td>0.630</td>
<td>++</td>
<td>0.150</td>
<td>-</td>
<td>0.236</td>
<td>+++</td>
</tr>
<tr>
<td>10188</td>
<td>0.201</td>
<td>+</td>
<td>0.044</td>
<td>-</td>
<td>0.012</td>
<td>-</td>
</tr>
<tr>
<td>12344</td>
<td>0.616</td>
<td>+</td>
<td>0.201</td>
<td>-</td>
<td>0.013</td>
<td>-</td>
</tr>
<tr>
<td>14769</td>
<td>0.594</td>
<td>++</td>
<td>0.182</td>
<td>-</td>
<td>0.077</td>
<td>-</td>
</tr>
<tr>
<td>12755</td>
<td>0.711</td>
<td>++</td>
<td>0.150</td>
<td>-</td>
<td>0.022</td>
<td>-</td>
</tr>
<tr>
<td>16874</td>
<td>0.942</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>0.044</td>
<td>-</td>
</tr>
<tr>
<td>9244</td>
<td>0.773</td>
<td>+++</td>
<td>0.202</td>
<td>-</td>
<td>0.52</td>
<td>++</td>
</tr>
<tr>
<td>14495</td>
<td>0.893</td>
<td>+++</td>
<td>0.547</td>
<td>-</td>
<td>0.298</td>
<td>-</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7458</td>
<td>0.009</td>
<td>-</td>
<td>0.016</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>7459</td>
<td>0.276</td>
<td>+</td>
<td>0.016</td>
<td>-</td>
<td>0.080</td>
<td>-</td>
</tr>
<tr>
<td>7460</td>
<td>0.330</td>
<td>+</td>
<td>0.203</td>
<td>-</td>
<td>0.349</td>
<td>+</td>
</tr>
<tr>
<td>7461</td>
<td>0.072</td>
<td>-</td>
<td>0.017</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>7462</td>
<td>0.051</td>
<td>-</td>
<td>0.017</td>
<td>-</td>
<td>0.013</td>
<td>-</td>
</tr>
<tr>
<td>7463</td>
<td>0.185</td>
<td>+</td>
<td>0.348</td>
<td>-</td>
<td>0.001</td>
<td>-</td>
</tr>
<tr>
<td>7464</td>
<td>0.143</td>
<td>-</td>
<td>0.005</td>
<td>-</td>
<td>0.032</td>
<td>-</td>
</tr>
<tr>
<td>7465</td>
<td>0.159</td>
<td>+</td>
<td>0.015</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>7466</td>
<td>0.065</td>
<td>-</td>
<td>0.088</td>
<td>-</td>
<td>0.022</td>
<td>-</td>
</tr>
<tr>
<td>7467</td>
<td>0.187</td>
<td>+</td>
<td>0.103</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 13: ELISA vs Western Blot anti-IgM results of 9 IM+ sera and 10 normal sera. WB = western blot. The number of (+) denotes the level of intensity of the western blot signal. The values in bold denote the highest ELISA value for which the corresponding WB result was negative and the next highest ELISA result for which the corresponding WB result was positive, for each recombinant protein.

For anti-IgM ELISA results any value greater than 0.159 (serum 7465) was positive for rVCApl8, for rEBNA1 all the western blot results were negative yet an OD reading up to 0.547 was found for one particular serum (14495). This would indicate that any OD reading equal or less than 0.547 may be considered negative. This reading was not used for establishment of a cut-off point as there were no other sera negative by western blot which had a comparable OD reading by ELISA. For rEA any OD reading above 0.349 was positive (serum 7460). Combining these results, a minimum OD reading @ 414nm of 0.349 or lower would indicate a negative result. As no rEBNA1 IgM-positive sera
were found it cannot conclusively be stated that any reading above this value would be positive.

<table>
<thead>
<tr>
<th>Serum</th>
<th>IgG Response</th>
<th>IgM Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p18VCA</td>
<td>EBNA1</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7435</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7436</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7438</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7439</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7442</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7443</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7447</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7448</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7449</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7454</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7456</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7457</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7458</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7459</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7460</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7461</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7462</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7463</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7464</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7465</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7466</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7467</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 14: IgG and IgM responses of normal sera to each of the recombinant antigens based on the reference value determined. (n/d - not done)
<table>
<thead>
<tr>
<th>Serum</th>
<th>IgG Response</th>
<th>IgM Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p18VCA</td>
<td>EBNA1</td>
</tr>
<tr>
<td>IM+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7083</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10035</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10188</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12344</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14769</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12755</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16874</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9244</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14495</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>15941</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13307</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12707</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15038</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15386</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11531</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6370</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6925</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9336</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12468</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12726</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 15: IgG and IgM responses of IM+ sera to each of the recombinant antigens based on the reference value determined.
<table>
<thead>
<tr>
<th>Serum</th>
<th>EBNA IgG Response</th>
<th>Serum</th>
<th>EBNA IgG Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA-</td>
<td></td>
<td>EBNA+</td>
<td></td>
</tr>
<tr>
<td>6985</td>
<td>+</td>
<td>7467</td>
<td>-</td>
</tr>
<tr>
<td>6627</td>
<td>-</td>
<td>8090</td>
<td>+</td>
</tr>
<tr>
<td>8694</td>
<td>-</td>
<td>7343</td>
<td>+</td>
</tr>
<tr>
<td>7127</td>
<td>-</td>
<td>7256</td>
<td>+</td>
</tr>
<tr>
<td>6988</td>
<td>-</td>
<td>7387</td>
<td>+</td>
</tr>
<tr>
<td>7068</td>
<td>-</td>
<td>7344</td>
<td>-</td>
</tr>
<tr>
<td>6737</td>
<td>-</td>
<td>7248</td>
<td>+</td>
</tr>
<tr>
<td>6891</td>
<td>-</td>
<td>7529</td>
<td>+</td>
</tr>
<tr>
<td>8725</td>
<td>-</td>
<td>7310</td>
<td>+</td>
</tr>
<tr>
<td>6769</td>
<td>+</td>
<td>7456</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 16: IgG response of EBNA-/+ sera to rEBNA1 based on the reference value determined.
<table>
<thead>
<tr>
<th>Serum</th>
<th>VCA IgM Response</th>
<th>Serum</th>
<th>VCA IgM Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCA-</td>
<td></td>
<td>VCA+</td>
<td></td>
</tr>
<tr>
<td>7523</td>
<td>-</td>
<td>8415</td>
<td>+</td>
</tr>
<tr>
<td>7268</td>
<td>-</td>
<td>8411</td>
<td>+</td>
</tr>
<tr>
<td>7274</td>
<td>-</td>
<td>7611</td>
<td>+</td>
</tr>
<tr>
<td>7291</td>
<td>-</td>
<td>7617</td>
<td>+</td>
</tr>
<tr>
<td>7299</td>
<td>-</td>
<td>7739</td>
<td>-</td>
</tr>
<tr>
<td>7300</td>
<td>-</td>
<td>7837</td>
<td>+</td>
</tr>
<tr>
<td>7266</td>
<td>-</td>
<td>8128</td>
<td>+</td>
</tr>
<tr>
<td>7271</td>
<td>-</td>
<td>8318</td>
<td>+</td>
</tr>
<tr>
<td>7281</td>
<td>-</td>
<td>8413</td>
<td>+</td>
</tr>
<tr>
<td>CMV+</td>
<td></td>
<td>EBV+</td>
<td></td>
</tr>
<tr>
<td>NY1</td>
<td>-</td>
<td>MS8</td>
<td>+</td>
</tr>
<tr>
<td>NY2</td>
<td>-</td>
<td>MS9</td>
<td>+</td>
</tr>
<tr>
<td>NY3</td>
<td>-</td>
<td>CAC6385</td>
<td>+</td>
</tr>
<tr>
<td>NY4</td>
<td>-</td>
<td>1318/95</td>
<td>-</td>
</tr>
<tr>
<td>NY5</td>
<td>-</td>
<td>26637</td>
<td>+</td>
</tr>
<tr>
<td>10190</td>
<td>+</td>
<td>2664</td>
<td>-</td>
</tr>
<tr>
<td>607/95</td>
<td>+</td>
<td>2656</td>
<td>-</td>
</tr>
<tr>
<td>8638</td>
<td>-</td>
<td>331/95</td>
<td>+</td>
</tr>
<tr>
<td>16792</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>69/95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0124</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1623</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>765/95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>769/95</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 17: IgM response of VCA-/+; CMV+ and EBV+ sera to rVCAp18 based on the reference value determined.
3.4.5 Cross-Reactivity Testing

In order to check for cross-reactivity with serum from patients with a different type of viral infection, a number of CMV-positive sera were tested with each of the recombinant EBV antigens by ELISA (see figure K). A number of EBV+ sera were also tested at this point. Sera which gave a positive result were then analysed by western blot also to determine if the positive reaction was due to reaction with the particular antigen or due to a high background reaction.

Figure K

Anti-p18VCA IgM Responses of EBV+ Sera and CMV+ Sera

Of 8 EBV positive sera tested, 5 showed a positive IgM response to p18VCA. 4 of 14 CMV-positive sera were also positive, by ELISA. The three EBV+ sera showing a negative anti-p18VCA IgM response (according to the reference point established in section 3.4.4) showed ELISA OD values of 0.225, 0.074, and 0.105, considerably lower than the cut-off point of 0.349. The four CMV+ sera showing a positive result were tested by western blot for IgM responses to each of the recombinant antigens.
Figure 46: Western blot analysis of purified recombinant proteins with CMV-positive sera and IM positive sera, probed with anti-human IgM POD conjugate. Lane 1 - protein markers, lanes 2-7 were probed with 6 CMV-positive sera (10190, 607/95, 0124, 0623, NY1 and NY2 respectively), lanes 8,9 were probed with 2 IM-positive sera (7083 and 16874).

Two of the CMV+ sera showed a very strong IgM response to rVCAp18 (sera 0124 and 0623, lanes 3 and 4, figure 46) which compares on intensity to the response of the positive control IM+ sera (7083 and 16874, lanes 8 and 9). The ELISA reading for these sera were 1.127 and 0.798 respectively. It cannot be determined from this result whether the reaction is due to EBV-specific antibodies or cross-reactivity of CMV-specific antibodies with the EBV recombinant antigens.
3.5 Discussion

3.5.1 Purification of the Fusion Proteins

A number of problems were encountered during purification of the recombinant antigens by osmotic shock, heat treatment and ammonium sulphate precipitation (sections 3.3.2.1, 3.3.2.2 and 3.3.2.3 respectively). Initially it was thought that osmotic shock would prove a convenient method of purification. Native thioredoxin is normally localised at the adhesion zones (Bayers patches), osmotically sensitive cellular compartments on the cytoplasmic side of the inner membrane. However, this did not appear to be the case for the thioredoxin fusion proteins; the recombinant proteins were not released to the shockate after osmotic shock treatment. Therefore, osmotic shock could not be used as a method of purification of the thioredoxin fusions, unlike thioredoxin. Heat treatment did allow the purification of the recombinant fusion proteins, however there were substantial losses. After 2 mn incubation @ 80°C a substantial amount of both the native proteins and the fusion protein was precipitated, a 10 mn incubation time was required to precipitate the majority of the *E. coli* proteins. This resulted in the majority of the fusion protein being precipitated also, leaving a small amount of soluble fusion protein. There was also native cellular protein remaining. Using ammonium sulphate precipitation there were no significant losses of fusion protein but the remaining *E. coli* proteins resulted in cross-reactivity in western blotting (data not shown), which could significantly interfere with ELISA assay or dot blot analysis of the recombinant antigens. Filtration through a centrifugal device with a 50 kD/100 kD cut-off was attempted after ammonium sulphate precipitation, to separate the residual cellular proteins, however it was found that all proteins were retained on the filter (data not shown). Affinity purification with PAO agarose was a compromise between loss of recombinant fusion protein and elimination of cellular *E. coli* proteins. Under the conditions used there was a limit to the amount of thioredoxin fusion protein which bound the column, a substantial amount of the fusion protein washed through the column, however this method did successfully eliminate the majority of the native *E. coli* proteins.

There was no reactivity observed when western blotting was carried out with thioredoxin and a number of IM+ sera (section 3.4.2.1, figure 41), therefore it was not necessary to cleave the thioredoxin moiety using enterokinase. This could be done after purification.
with PAO agarose, however it is possible that thioredoxin lends stability to the fusion protein. As this cleavage was not essential it was preferred not to further manipulate the recombinant proteins which could result in further losses in yield. This could also result in degradation due to incubation at 37°C, possibly for a number of hours, depending on the nature of the fusion protein. The digestion conditions would also have had to be optimized for each protein, possibly without advantage gained with regard to the usefulness of the recombinant protein as a diagnostic reagent.

In order to purify the proteins to homogeneity subcloning may be required. A number of cloning vectors are available which also allow the expression of fusion proteins in *E. coli*. This original Thiofusion system has recently been improved to allow easier sub-cloning and simplified purification. The vector used for expression contains a modified thioredoxin gene that allows purification of fusion proteins on metal-chelating resins (Invitrogen catalog 1998). A number of other examples include Glutathione-S-Transferase (GST) (Wang *et al.*, 1997, Bettadapura *et al.*, 1998), Maltose Binding Protein (MBP) (di Guan *et al.*, 1987) and Chitin Binding Domain (CBD) (Chong *et al.*, 1997, Rozynek *et al.*, 1997), fusions. These fusion proteins allow purification by affinity column, which would be more specific than the phenylarsine oxide (PAO) agarose column used for thioredoxin purification, this may also improve the yield of recombinant protein. For example, using a column with maltose as affinity tag in the case of MBP. Alternatively, the existing system may be used with further purification of the proteins already partially purified by PAO agarose. High performance liquid chromatography (HPLC), is a possible purification alternative, which allows complete purification to homogeneity (Oliver 1989), reverse-phase HPLC may be appropriate for the purification of these recombinant proteins. However, this method is very expensive in comparison to those used to date.
3.5.2 Loss of Expression of rBARF0

As the BARF0 ORF encodes a novel EBV protein which may be of value in the diagnosis of NPC, it was chosen for expression in *E. coli* using the Thiофusion expression system. Initial expression of the rBARF0 fusion protein was quite strong, however, it was found that the selected clones could not be induced to express this fusion protein on a larger scale (400 ml culture). Restriction analysis was carried out on purified plasmid DNA, which showed that the insert (of the correct size) was still present and in the correct orientation. Freshly prepared competent *E. coli* cells (G1724 strain) were transformed with the recombinant plasmid. Induction of both small and large scale cultures showed that there was no fusion protein expressed. It is possible that there was a deletion mutation within the DNA sequence of the BARF0 ORF at some stage during propagation. If the mutation was only one base it could not be detected by restriction analysis unless it occurred at one of the chosen restriction sites. This may have caused a frameshift in the DNA sequence which would result in the gene being out of frame thus preventing translation of the fusion protein. If this were the case there would be no visible expression of rBARF0. However, as this protein was expressed during the initial growth and induction it does not seem likely. It is also possible that the mRNA was unstable, or the fusion protein itself was unstable. Mutation or instability might have caused a loss of expression due to selection against rProtein expressing cells. In order to regain expression of this fusion protein, it would be necessary to re-amplify the BARF0 ORF, possibly changing one or both primers. The new PCR product would then be ligated with the pTrxFus vector as before and selected clones analyzed for fusion protein expression. Cloning in an alternative vector may also be necessary.

3.5.3 Background ELISA Signal

When the three proteins were combined and analyzed by western blot there was no apparent non-specific signal detected (figures 63 and 64), however ELISA analysis did result in significant background signals possibly due to the difference in the mode of antigen presentation and assay conditions. The conditions were varied (method of immobilization, blocking buffer, washing stringency, number of washes, antigen concentration, secondary antibody concentration and serum dilution factor) in order to minimize the background signal but a low level remained consistently (data not shown).
The *E.coli* G1724 PAO purified cell extract used in ELISA reactions to evaluate the background signal could be pre-incubated with the diluted serum before addition to the antigen coated wells. In this way the signal obtained (optical density reading @ 414nm) would not require manipulation to account for reactivity of serum antibodies with native *E.coli* proteins. Further purification (as discussed in section 3.5.1) might also significantly reduce the background signal by removal of the native cellular proteins.

The recombinant protein concentration used for coating the ELISA wells was 10 µg/ml and the concentration of background proteins was 8.4 µg/ml, therefore the concentration of the specific protein being analysed was only a little greater than the background protein. The level of reactivity to the native proteins was higher than expected, being greater for anti-human IgG than anti-human IgM. This would be expected as IgM antibodies are only present at elevated levels in the initial stages of an infection. Assuming the serum samples tested were from patients without any other infection (apart from EBV in the cases of 'IM+', 'EBV+' and 'VCA+' sera) their non-specific IgM antibody levels should be very low. It is possible that a low level of IgG antibodies to a number of *E.coli* proteins present in the G1724 extract and the recombinant protein preparations, could produce a signal which would significantly amplify the apparent anti-EBV antibody response.

Five percent foetal calf serum (FCS) was used as diluent for the serum sample. The antibodies present in the FCS could bind the *E.coli* proteins thus reducing the level of human antibodies reacting with the bacterial proteins. However, the difference in background signal did not vary significantly from that obtained when using 5% BSA as diluent. Changing the blocking buffer from 5% BLOTTO to 5% BSA, and increasing the concentration of Tween (0.1%) in the washing buffer also aided the reduction in the background signal.

Initially an anti-thioredoxin (α-Trx) antibody was used to immobilise the fusion proteins, however it was found that the anti-thioredoxin antibody was reacting with the human serum and gave a significant reaction with the blocking buffer also (data not shown).
Coating with α-Trx, without the addition of recombinant protein, followed by incubation with serum gave an OD reading which compared with that obtained when recombinant protein or thioredoxin was also added. It is not certain why this occurred, but may be due to the presence of contaminating antibodies in the α-Trx antibody preparation. It was necessary to directly immobilise the recombinant antigen preparation on the ELISA plate. Altering the antigen concentration and the secondary antibody concentration did not cause a reduction in the background signal (data not shown), the net difference between the recombinant antigen OD reading and that of the G1724 cell extract remained constant. In order to confirm the data obtained, it would be necessary to use fully purified recombinant proteins to eliminate the existence of non-specific interactions due to the presence of *E. coli* proteins.

3.5.4 Reference Point for Evaluation of ELISA Data

The establishment of a reference point was essential in evaluation of the ELISA data. All of the sera tested had been characterised by an alternative EBV diagnostic method, except for the normal sera (serum samples for routine clinical investigations) which had not been assayed for anti-EBV antibodies. As all the methods used were different, it was necessary to establish another specific method of evaluation of a number of sera of different types for direct comparison with the ELISA data. A number of normal sera and IM+ sera were chosen for western blot analysis. The lowest OD reading by ELISA above which there was no negative result by western blot was the chosen reference point for negative/positive ELISA results. There were a number of sera with ELISA OD readings lower than the reference point, which showed a positive western blot result. One figure was chosen for IgG responses and one for IgM responses.

There were a number of ways in which such a reference point could be determined. All of the sera analysed could be tested using one specific EBV diagnostic method, whose results are considered conclusive. Immunofluorescence assay (IFA) is often the chosen method for comparison with new screening methods (Wiedbrauk and Bassin, 1993). IFA is the standard method for detection of anti-VCA antibodies, however it is a difficult assay to standardise, time-consuming and not suitable for large scale screening.
Heterophile antibody testing could also be used but this test is not reliable for the testing of serum from children (Lennette et al., 1991) Therefore the results could not be considered 100% reliable as the age of the patients whose serum was used was not known in the majority of cases.

The difficulty in determining conclusive results with EBV testing is that only a very small percentage of the population are EBV negative. It would be necessary to use sera from candidates who are seronegative for EBV and those who are conclusively IM+ (diagnosed by a number of tests and displaying the relevant symptoms) in order to determine the limitations of these novel recombinant EBV antigens. Very young infants are the only reliable source of EBV-negative serum.

To confirm the effectiveness of these antigens for diagnosis of IM or other EBV-related diseases it would be necessary to screen a larger panel of serum samples in parallel with commercial kits. Analysis by western blot and an alternative anti-EBV ELISA could be used to determine the sensitivity and specificity of this method. Commercial ELISAs have proven to provide a reproducible method for the serological diagnosis of infectious mononucleosis, however, the different tests can show considerable variation in performance based on the different methods and reagents used, specifically the different antigens used (Weber et al., 1996, Gutiérrez et al., 1997, Mitchell et al., 1998).

3.5.5 Antibody profiles using the recombinant EBV antigens

Using the reference point determined by comparison of western blot results and ELISA results, each of the serum samples were designated positive or negative for the recombinant antigens tested. For the majority of the samples the results were similar to those expected. As patient age, history and symptoms (if present) were not known it was difficult to critically assess the possible reasons for any deviations from the expected profile.
The normal sera tested had not previously been tested for anti-EBV antibodies. The Monosticon DRI-DOT (Heterophile antibody) test was used to assess a number of the samples, all proved negative for the presence of heterophile antibody. Only one normal serum, 7460, showed a positive IgM response to EA there was no response to any of the recombinant antigens for the remainder of the sera tested for anti-IgM, however the anti-IgG profile varied. Fifteen of 22 sera were EBNA1 positive, of these 15, 10 were also p18VCA positive. Two serum samples showed a positive IgG response to p18VCA but a negative response to EBNA1, the majority were EA-D-negative (17/22) as expected. In the case of the 5 samples which were EBNA1 and p18VCA-negative (also EA-D-negative), this would indicate the absence of an EBV infection. Those showing both EBNA1 and p18VCA-positive IgG responses may have had a previous, active infection resulting in the persistence of an anti-p18VCA IgG response. For those who were EBNA1-positive but p18VCA-negative for IgG, this would suggest the presence of a latent EBV infection. The presence of anti-p18VCA IgG antibodies in the absence of anti-EBNA1 IgG is not to be expected. As immune responses vary between individuals it might be that certain individuals have a higher p18VCA titer than EBNA1 titer. It is possible, although unlikely for normal serum samples, that these patients were in the convalescent stage of IM, if the infection was at an acute stage an anti-p18VCA IgM response would also be expected. The reference point for determining positive or negative results may be too high to detect low anti-EBNA1 IgG responses, therefore, these individuals could also have had a low level of anti-EBNA1 IgG antibodies. Eighty two percent of the normal sera appeared to be EBV seropositive, this is close to the expected percentage for the general population (~ 90%), while 100% were IgM-negative.

Sixteen of 19 of the IM+ sera tested, were shown to be positive for anti-p18VCA IgM, this would be indicative of an acute IM infection. There were no IgM responses to EBNA1 or EA-D, except for serum 14495, which showed an IgM response to EBNA1. The OD reading for this sample was considerably higher than the other EBNA1 IgM readings and was not taken into consideration when establishing the reference point (section 3.4.4). Of the 16 p18VCA IgM-positive samples, 3 were also anti-p18VCA IgG-positive, in these cases the infection may have progressed by 3-4 weeks at which
stage the IgG response to p18VCA is expected to peak. All 19 sera were anti-EBNA1 IgG-negative which is characteristic of EBV-induced IM. Six of 19 samples were anti-EA-D IgG-positive, only one of these was also p18VCA IgG-positive. The EA-D IgG response peaks at approximately the same time as the p18VCA IgG response but anti-EA-D IgG antibodies only appear in 80% of infected individuals. As the majority of the samples were p18VCA IgM-positive and p18VCA IgG-negative it is most likely that the samples were taken at the acute stage of IM before the development of p18VCA and EA-D IgG antibodies. Of the 11 sera tested with the Monosticon DRI-DOT kit, 7 were positive and 4 were negative. All of the positive samples were also p18VCA IgM-positive, and 2 of those which were negative were also p18VCA IgM-and IgG-negative. However, the other two samples which were negative for the heterophile antibody test were positive for p18VCA IgM (15941 and 11531). The OD reading for each serum was well above the point cut-off point (0.349), 15941 (OD @ 414nm = 0.686) and 11531 (OD @ 414nm = 0.806). If these individuals were children (less than 10 yrs) it is possible that the heterophile antibody test could show a false-negative result (Lenette, 1991).

Seven of 10 EBNA+ sera were EBNA1 IgG-positive by ELISA. The BIOTEST anti-EBV test kit used a 46 kD recombinant EBNA1 which is considerably larger than the truncated form used here (29 kD, does not have the gly-ala repeat sequence, see figure 12). The larger recombinant protein would encompass a greater number of epitopes, it is possible that those epitopes were not present in the truncated protein, hence the negative response in the case of 3 serum samples. It is possible that this test, with the truncated recombinant EBNA1, is more specific due to the absence of the gly-ala repeat sequence which may result in false-positive results (Milman et al., 1985). Two EBNA- sera showed a positive response (OD - 0.394 and 0.302), both were close to the cut-off point for IgG responses (0.302). As the recombinant EBNA1 was fused to thioredoxin, this may be the result of non-specific interaction with the thioredoxin moiety. However, increasing concentrations of free thioredoxin were added to serum samples analysed by ELISA, with no reduction in the ELISA signal (data not shown). This would indicate the absence of any thioredoxin-specific antibody response.
All VCA-negative sera were also negative for p18VCA IgM by ELISA. Two of 9 VCA+ sera were negative for p18VCA IgM. This may be due to the fact that the Gull VCA IgM ELISA was based on the EBV gp125 glycoprotein. As the antigen was different it is quite possible that the test results would also differ. Individuals elicit antibody responses to different epitopes, it may be that these conflicting results are a consequence of this phenomenon. The Baxter Bartels EBV IgM EIA was also based on IgM responses to the EBV gp125 glycoprotein, only 5 the 8 sera characterised as EBV+ by this assay were also positive for anti-p18VCA IgM by ELISA. As with the VCA+ sera this may be due to differences in antigen presentation and epitope recognition in each individual. Loss of specific antibodies due to storage/freeze-thawing may also have been a factor here. Differences in other reagents and test procedures may also account for the observed inconsistencies.

3.5.6 Cross-reactivity with Sera from CMV-Positive Individuals

Cytomegalovirus (HHV5) is a member of the betaherpesviriniae subfamily, and it’s genomic structure is very different to that of EBV (a member of the gammaherpesviriniae subfamily). This virus causes a mononucleosis-like syndrome also, and can be maintained in a latent form in lymphoreticular cells, secretory glands, kidneys and other tissues. Human CMV is often isolated from apparently normal human adenoids and salivary glands. (IARC Monographs 1998). Fourteen CMV+ sera were tested for IgM antibody to rVCAp18 by ELISA for cross-reactivity of IgM antibodies. Anti-rVCAp18 IgM was the chosen test as this response appears to be of the most significant diagnostic value in relation to acute EBV-IM infection (in this assay system). A positive reaction would be indicative of an acute IM infection, a case of cross-reactivity between CMV-specific antibodies and rVCAp18 or reactivity with the background E.coli proteins. It was found that of 14 samples, 4 showed a positive reaction with rVCAp18 (i.e. an OD reading @ 414nm greater than 0.349) i.e. 29% were cross-reactive. Those giving a positive reaction were subsequently analysed by western blot for IgM antibodies to all three recombinant antigens. Two sera showed a strong response to rVCA while the other two showed no specific IgM responses. In general the level of background signal was high for each of the 4 serum samples and also for one serum sample which had shown a negative rVCAp18 IgM response.
It cannot be confirmed from these results whether the IgM response to \( rVCAp18 \) was a cross reaction of CMV specific IgM antibodies. It is possible that the 'cross reacting' samples were taken from individuals with coincident EBV primary infection. It might also be the case that the two ELISA +/Blot- sera exhibited non-specific IgM responses with the native cellular proteins in the EBV p18 antigen preparation. The samples showing a positive IgM response to \( rVCAp18 \) by western blot may have specific anti-p18VCA antibodies, however this may be a clear case of cross-reaction between anti-CMV antibodies and \( rVCAp18 \). In order to clarify these results it would be necessary to simultaneously test these sera by gp125 and p18VCA ELISA and western blot. Also pre-incubation of the sera with total CMV purified antigens, prior to assay by ELISA or western blot for EBV would allow adsorption of CMV-specific antibodies. If the assay results were still positive this would indicate an EBV-specific antibody response, if the result was negative it would seem that the CMV-specific antibodies were cross-reacting with \( rVCAp18 \). This could also be done in reverse, by pre-adsorption with EBV antigen and subsequent testing for the presence of CMV-specific antibodies. It would also be necessary to test other serotypes against these recombinant antigens, such as HSV, adenovirus, varicella zoster and HIV-positive individuals.

Cross-reactivity and false-positive results are problems which must be eliminated in any diagnostic test system. If the specificity of the assay is low the level of misdiagnosis would be too great to allow the commercial availability of such a diagnostic kit. This further emphasises the need for complete purification of the recombinant antigens. It may also be necessary to cleave the thioredoxin moiety from the fusion proteins to further reduce the possibility of cross-reactivity and the appearance of false-positive results. However, as was shown on western blot analysis, figures 44-47, many sera show reactivity with just one antigen, or none of the antigens. If there was a specific interaction with thioredoxin this would be evident for each antigen.

### 3.5.7 Expression of other recombinant EBV antigens

The EBV glycoprotein gp125 (gp125 is part of the VCA complex) has been used in the development of many diagnostic methods, this glycoprotein can be purified from P3HR-1.
cells in culture. The three commercial tests used for partial characterisation of the sera analysed here, IFA, VCA IgM ELISA (both from Gull Laboratories) and a second VCA IgM ELISA (Baxter Bartels) all use gp125 purified from P3HR-1 cells. This protein has also been expressed in the baculovirus expression system and has been tested with IM+ sera for IgG antibodies in an immunoblot assay, in comparison with anti-VCA IFA (Sanchez-Martinez et al., 1995). The assay showed 97% sensitivity and 100% specificity, with no crossreactivity with other human herpesviruses. It may also be possible to express the gp125 glycoprotein in the Thiofusion expression system, however, the absence of the carbohydrate residues is likely to influence the immunogenicity. However, as the gp125 shares significant homology with other herpesvirus glycoproteins, cross-reactivity cannot be excluded in all test systems (van Grunsven et al., 1994).

Other recombinant EBV antigens have also been evaluated as diagnostic reagents for NPC, these include DNA polymerase (DP), in E coli (Lin et al., 1995), EBV DNase, thymidine kinase (TK) and gp350/220 in baculovirus or bovine papillomavirus (Littler et al., 1991). IgG antibodies to DP were detected in 43 of 48 NPC-positive sera, without any crossreactivity with CMV or HSV-1. Thymidine kinase was shown to be the most sensitive marker for NPC in comparison with DNase and gp350/220. Recombinant EBV DNA binding protein (DBP), p138, has been shown to be effective in diagnosis of IM in conjunction with other antigens, i.e. EA-D, VCA and EBNA1 (Gorgievski-Hrisoho et al., 1990). Thymidine kinase, DBP (p138) and DP are also candidates for expression in the Thiofusion expression system.

No one EBV antigen is sufficient to diagnose the type or stage of all types of EBV infection. However, a panel of antigens already expressed by the thiofusion expression system, and expression of a number of other antigens such as TK, DBP, gp125, and DP by this method, could provide a selection of the most diagnostically sensitive proteins, for an effective means of diagnosis of many EBV-related diseases.
3.5.8 Future Recommendations

* Further purification of each of the fusion proteins - either by sub-cloning into a vector which allows an alternative method of affinity purification, or using more effective purification procedures such as reverse-phase HPLC

* Testing of a larger panel of sera by several serology-based methods - based on specific antibody responses, this would provide a consistent reference for the results of this ELISA method using the recombinant antigens

* Testing a broader range of sera - from individuals whose age, medical history and symptomology is known, this would allow more informed critical analysis of the test results

* Testing of sera from patients at different stages of EBV-induced IM - acute, convalescent and re-activation for whom medical data is available

* Testing of a number of serum samples from one patient with IM - samples taken at different stages during the progression of the disease from initial diagnosis through to convalescence

* Analysis of sera from patients with other EBV-related diseases - such as Burkitt’s lymphoma (BL), Hodgkin’s disease (HD) and Nasopharyngeal carcinoma (NPC), also, immunocompromised individuals (HIV+ and Post-transplant patients)

* Testing of sera from patients with other non-EBV viral infections - such as HSV, adenovirus and varicella zoster

* Assay for anti-p18VCA IgA and anti-EA-D IgA antibodies - with sera from IM re-activation patients and NPC+ individuals

* Testing of saliva samples from NPC+ and NPC- individuals - detection of anti-EA-D IgA antibodies in saliva has been proven effective in the diagnosis of NPC (Nadala et al., 1996)

* Development of a more rapid assay format - possibly using a membrane based system
Chapter 4

Identification of anti-gp350 MAb-binding peptides using phage display.
4.1 Introduction

The most abundant protein of the EBV envelope is the glycoprotein gp350/220. This protein mediates virus adsorption and penetration of the host cell via the complement receptor molecule CR2. A number of anti-gp350/220 monoclonal antibodies prevent virus infection and abolish its ability to transform B cells. As gp350/220 can induce antibodies which can both neutralise the virus and inhibit its binding to target cell receptors, it has been the focus of EBV vaccine preparations. The monoclonal antibody 72A1, an IgG2a class murine antibody, has been shown to neutralise both type A and type B strains of EBV via binding with gp350/220 (Sairenji et al., 1988). It is thought that the epitope recognised by 72A1 is the only glycoprotein domain with a dual role in EBV neutralisation and binding to target cell receptors (Stocco et al., 1990). If the epitope recognised by 72A1 were known it might then be possible to develop a subunit vaccine with this peptide as a component. In this way a vaccine would both neutralise the virus and simultaneously prevent binding of the virus to its target receptor. The purpose of the following experiments was to identify peptides that bind to 72A1 and also the monoclonal antibodies F29 167 and F2 1, both IgG2a class murine antibodies that are also capable of virus-neutralisation, by screening random peptide phage libraries.

Phage display libraries have been used in many instances to identify the epitopes of a particular antigen as discussed in section 1.8. Although this is mostly successful with epitopes known to be continuous, it is also possible to identify discontinuous epitopes. The type of library and the nature of the antibody are both significant factors in the success of epitope mapping. The preference for a given library in yielding the best-binding peptides, depends on the structural framework within which a random peptide is presented, and so the chances of success are increased. Four libraries were chosen for screening with the anti-gp350/220 monoclonal antibody 72A1, these are summarised in table 18 and described in turn below.
4.1.1 The Random Phage Peptide Libraries (RPLs)

<table>
<thead>
<tr>
<th>Library</th>
<th>Vector Type</th>
<th>Peptide length (amino acids)</th>
<th>Complexity (transformants)</th>
<th>Concentration (virions/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIII 15 mer</td>
<td>fUSE5</td>
<td>15</td>
<td>1.1*10^{12}</td>
<td>10^{14}</td>
</tr>
<tr>
<td>pIII 7 mer</td>
<td>M13</td>
<td>7</td>
<td>2*10^{9}</td>
<td>2*10^{13}</td>
</tr>
<tr>
<td>pIII 12 mer</td>
<td>M13</td>
<td>12</td>
<td>1.9*10^{9}</td>
<td>4*10^{12}</td>
</tr>
<tr>
<td>pIII 7 mer constrained</td>
<td>M13</td>
<td>7</td>
<td>3.7*10^{9}</td>
<td>2*10^{13}</td>
</tr>
</tbody>
</table>

Table 18: Phage display libraries.

The 15 mer library was constructed in vector fUSE5 (Scott and Smith, 1990), which was derived from the tetracycline resistant filamentous phage fd-tet. Vectors which are derivatives of fd-tet do not kill their host, probably due to a reduced RF (replicative form) copy number that is caused by a defect in minus-strand DNA synthesis. As a result, these derivatives have less than 5% infectivity as compared to 50% in wild type filamentous phage. fUSE5 differs from other fd-tet derivatives in having two SfiI sites with different overhanging 3-base 3' ends, and a frameshift mutation in gene III 2-3 amino acids downstream of the signal sequence. The library was constructed by splicing degenerate oligonucleotide inserts into the SfiI sites, thus restoring the reading frame, leading to a recombinant pIII that is incorporated as a ring of five molecules at one tip of the virion. The presence of a peptide insert restores the ORF and the majority of gene III is uninterrupted by inserts. As this vector does not kill it’s host the demand for pIII function is minimised (pIII is required for both infection and morphogenesis). Therefore, there might not be a strong selection against inserts which impair pIII, during propagation. The K91Kan Escherichia coli strain was used for propagation of these phage, this strain is kanamycin resistant, and upon infection with phage it also acquires tetracycline resistance. Successfully infected host bacteria can then be selected on media with both kanamycin and tetracycline. This library, a gift from Dr.A.J.Conley (University of Missouri), was supplied as a secondary amplification of the primary library yielding 1.1*10^{12} transductant clones.
The 7 mer library consisted of $2 \times 10^9$ electroporated sequences, amplified once to yield approximately 100 copies of each sequence in 10 µl of the phage. The displayed heptapeptides are expressed directly at the N-terminus of pIII, that is the first residue of the mature protein is the first randomised position. The peptide is followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild-type pIII sequence.

The 12 mer library consisted of approximately $1.9 \times 10^9$ electroporated sequences, amplified once to yield approximately 20 copies of each sequence in 10 µl of the phage supplied. Similar to the 7 mer library the peptides are expressed at the N-terminus of pIII, again followed by the 4 mer spacer and then the wild-type pIII sequence.

Unlike these libraries the random peptide of the 7 mer constrained library phage is flanked by a pair of cysteine residues. Under non-reducing conditions the cysteines will spontaneously form a disulphide cross-link, resulting in phage display of cyclised peptides. The peptide is expressed at the same position of the pIII protein as that of the 7 mer and 12 mer libraries, with the first cysteine being preceded by an alanine residue. This library consists of $3.7 \times 10^9$ electroporated sequences which when amplified yield approximately 50 copies of each sequence per 10 µl of supplied phage.

### 4.1.2 The Anti-EBV Monoclonal Antibodies

The antibodies used in biopanning are described in Table 19 below.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Subtype</th>
<th>Antigen</th>
<th>Virus neutralising capacity</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>72A1</td>
<td>IgG2a</td>
<td>gp350/220</td>
<td>with or without complement</td>
<td>360 µg/ml</td>
</tr>
<tr>
<td>F2 1</td>
<td>IgG2a</td>
<td>gp85</td>
<td>only with complement</td>
<td>500 µg/ml</td>
</tr>
<tr>
<td>F29 167</td>
<td>IgG2a</td>
<td>gp350/220</td>
<td>with or without complement</td>
<td>1.0 mg/ml</td>
</tr>
</tbody>
</table>

**Table 19:** Anti-EBV monoclonal antibodies.
4.2 Results

4.2.1 Biopanning of the RPLS

Primarily the 15 mer library was panned with both 72A1 and F21. Six amino acids is a typical size for a continuous epitopes, however, using a 15 mer library increases the effective size of the library, since each phage represents several short peptide determinants (e.g., up to 10 hexapeptides) in various contexts of flanking residues (Scott and Smith 1990). The longer peptide makes it possible to select epitopes comprising more than six residues, also the peptides displayed in libraries with longer randomized regions may have some structural features not present in ‘shorter’ libraries. The biopanning procedure used is detailed in section 2.6.5. This involved covalent linkage of biotin to the target molecule, this was done prior to incubation with the phage library. Phage able to bind the target were then selected by ‘panning’ the mixture of phage and target molecule on a streptavidin-coated dish. This exploited the strong biotin-streptavidin interaction, retaining the target molecule and consequently the phage bound to it. Bound phage were then eluted from the complexes and subjected to further rounds of amplification in E.coli and biopanning, three rounds of panning were carried out. See figure 47 for a flow diagram of this procedure.

A 7 mer, 12 mer and 7 mer constrained library were also screened. The 7 mer constrained library was chosen for screening as it has been shown that screening libraries which are constrained often prove to be a richer source of mimotopes than linear peptides expressed in an unconstrained format (Ladner et al., 1995). Constraining the peptide confers the advantage of restricting the peptide to fewer conformations, allowing the determination of more structurally complex epitopes. Two alternative procedures were used for these libraries.
Repeat twice

Biotinylate monoclonal antibody

↓

Coat plate with streptavidin

↓

Immobilise biotinylated antibody on streptavidin coated plate

↓

Add phage to the plate

↓

Wash away unbound phage

↓

Elute bound phage

↓

Titer eluate and amplify in *E. coli* K91Kan

Figure 47: Flow diagram of the biopanning procedure for the 15 mer phage library.

An alternative method was used in panning the 7 mer library from New England Biolabs (See section 2.5.3) In this case the antibody was immobilised without exploiting the streptavidin-biotin interaction, as a result a number of steps could be removed from the procedure thus simplifying the process. It was not necessary to biotinylate the antibody or to coat the plate with streptavidin. The remainder of the procedure is similar to that of the 15 mer library, figure 48 shows a flow diagram of the protocol.
Repeat twice

Repeat twice

- Immobilise monoclonal antibody directly on plate
- Add phage to the plate
- Wash away unbound phage
- Elute bound phage
- Titer and amplify in *E. coli* ER2537

**Figure 48:** Flow diagram of the biopanning procedure for the 7 mer phage library.

Both the 12 mer and 7 mer constrained libraries were panned via solution binding with protein G capture (See section 2.5.6) By this method the library was allowed to react with the antibody in solution, followed by affinity capture of the antibody-phage complexes onto protein G (protein A can also be used in this procedure) In addition to requiring substantially less antibody than surface panning, solution panning can result in improved accessibility of the antigen binding site to phage-displayed peptides, as well as avoiding partial denaturation of the antibody on the plastic surface A flow diagram of solution panning is shown in figure 49
Wash and block protein G agarose

Mix monoclonal antibody and phage

Immoblise antibody/phage mix on protein G

Wash away unbound phage

Elute bound phage

Titer eluate and amplify in *E. coli* ER2537

Figure 49: Flow diagram of the biopanning procedure for the 7 mer constrained phage library and the 12 mer library.

Three rounds of panning were carried out in each case and the eluate from each round was amplified. The third round was titered (sections 2.5.2 and 2.6.3) before amplification and single plaques were isolated for small-scale propagation and processing (sections 2.5.4 and 2.6.5)
4.2.2 Phage Titering

After each round of panning the phage, the eluate was titered before and after amplification to ensure that a sufficient number of phage had been selected during panning and that the amplification procedure has produced enough virions to proceed with to the next round (See sections 2.5.2, 2.6.3, and 2.6.4) The following tables show typical titering results after each round of panning of the 7 mer library with the monoclonal antibodies 72A1 and F29.167 and the 7 mer constrained library with 72A1

<table>
<thead>
<tr>
<th>Round no.</th>
<th>Amp. (−/+)</th>
<th>Phage titer (phage/μl)</th>
<th>Volume used (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72A1</td>
<td>F29.167</td>
<td>72A1</td>
</tr>
<tr>
<td>1</td>
<td>−</td>
<td>3*10²</td>
<td>6*10⁴</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>1 68*10¹⁰</td>
<td>5 52*10¹⁰</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>8*10³</td>
<td>6 3*10³</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>1 34*10¹⁰</td>
<td>2 2*10¹⁰</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>3 68*10⁵</td>
<td>10⁶</td>
</tr>
</tbody>
</table>

Table 20A: Titering results for the 7 mer library when panned with 72A1 and F29.167.

<table>
<thead>
<tr>
<th>Round no.</th>
<th>Amp. (−/+)</th>
<th>Phage titer (phage/μl)</th>
<th>Volume for next round (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>3*10²</td>
<td>−</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>2*10¹⁰</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>2*10²</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>2*10¹⁰</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>2 5*10²</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>3 3*10¹⁰</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 20B: Titering results for the 7 mer constrained library when panned with 72A1.

Theoretically the number of phage eluted in each round should increase if selection is occurring. As can be seen in tables 20A and 20B, the number of phage eluted does not
increase with increasing rounds of panning. However, amplification is successful in amplifying the number of phage eluted. It may be that the phage being amplified do not have high affinity for the selecting antibody, thus on subsequent rounds of panning there is no increase in the number of phage displaying 72A1-specific peptides. The concentration of monoclonal antibody used during biopanning is decreased with increased round number. This is done to increase the number of peptides selected which have a high affinity for 72A1 and minimise low affinity peptide selection. Therefore, the lack of increase in the number of phage selected may reflect the selection of only those peptides with high affinity for 72A1, and the loss of phage displaying peptides with low affinity for 72A1.

4.2.3 Sequencing of Selected Phage Clones

It was decided to proceed with sequencing of selected phage clones from each library regardless of the findings of the phage titers. Phage DNA was isolated from the selected plaques isolated from the third round and DNA sequencing template was prepared (sections 2.5.5 and 2.6.7). The template preparations were analysed by electrophoresis on 0.8% agarose gel (1X TBE) before proceeding with the sequencing reactions. Figures 50 and 51 show the DNA preparations for clones selected from the 7 mer, 12 mer and 7 mer constrained libraries. The sequencing reactions and sequencing gel were prepared and electrophoresed as described in section 2.7.
Figure 50: Analysis of phage template DNA prior to sequencing. Phage clones were selected from the eluate of the third round of panning of the 7 mer library panned with 72A1. Lanes are as follows, 1-4- DNA preps of phage clones selected from the 7 mer library with the anti-gp350/220 monoclonal antibody 72A1 (phage propagated in 15 ml microfuge tubes), 5-19- DNA preps of phage clones selected from the 7 mer library with the anti-gp350/220 monoclonal antibody F29 167, 20- DNA ladder

Figure 51: Analysis of phage template DNA prior to sequencing. Phage clones selected from the eluate of the third round of panning of the 12 mer library and the 7 mer constrained library panned with 72A1. Lanes are as follows, 1- DNA ladder, 2-6- DNA preps of phage clones selected from the 7 mer constrained primary (un-panned) library, 7-19- DNA preps of phage clones selected from the 12 mer primary (un-panned) library, 20- DNA ladder
Figure 52 shows a photograph of the developed autoradiograph, indicating the variable region (insert) for a number of the 15 mer clones selected with 72A1. The majority of sequences were clearly legible, as a control, M13 template DNA was also sequenced. In general the sequences of individual clones from each library were grouped according to similarities in amino acid composition and positioning. From the 15 mer library three distinct groups were observed, clones from the 7 mer library were divided into five groups, while the sequences found in the 7 mer constrained library were divided into just two groups. Sequencing of clones isolated from the 12 mer library yielded only phage with the native pIII protein sequence, i.e., no inserts present. It is possible, although unlikely, that the antibody selected these phage or that phage without inserts (or wild type contaminating phage) may have had a growth advantage over those with an insert in the pIII protein, and during amplification outgrew any phage specifically selected by the monoclonal antibody 72A1.

A small number of clones selected from the 15 mer library with the F21 monoclonal antibody were also sequenced. Tables 21-24 show lists and groupings of sequenced clones.
Figure 52: Photograph of the DNA sequencing autoradiograph, showing sequences from the 15 mer library. The sequencing reactions were run in the order A,C,G,T (left to right) Set 1- clone 1 1, set 2- clone 1 2, set 3- clone 2 4, set 4- clone 1 6
<table>
<thead>
<tr>
<th>Group no.</th>
<th>Clone no.</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>GP YKNHLWWKSPISRRLR SPV</td>
</tr>
<tr>
<td></td>
<td>111</td>
<td>GP FYHSHKWWVLPMPRG SPV</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>GP RHWWFSSAARHRPLRG SPV</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>GP RGWWTHVHTNRGWLA SPV</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>GP CTHLSRWGSRKANM SPV</td>
</tr>
<tr>
<td></td>
<td>212</td>
<td>GP KQRKHWFPSGPVSV SPV</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>GP RKWGFWQHVLQVHRDS SPV</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>GP CTHLSRWGSRKANM SPV</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>GP ESFLHVPRWASLVS R SPV</td>
</tr>
<tr>
<td></td>
<td>112</td>
<td>GP YRSWRGGWSSHLKGH SPV</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>GP YKNHLWWKSPISRRLR SPV</td>
</tr>
<tr>
<td>4</td>
<td>113</td>
<td>GP HSFYIHALTGGSMHR SPV</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>GP HCHRWSCREYRAQDR SPV</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>GP HCHRRCMSYARKDM SPV</td>
</tr>
<tr>
<td>Ungrouped</td>
<td>18</td>
<td>GP RSHFGHYKERVTRTRY SPV</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>GP HAIRSGKCRWWACMS SPV</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>GP GAFLKSFCHGFRPS SPV</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>GP SHKWHLYFGKPTRTK SPV</td>
</tr>
<tr>
<td></td>
<td>213</td>
<td>GP VLTRKHTDFWVIR SPV</td>
</tr>
<tr>
<td></td>
<td>214</td>
<td>GP RGDWRYGLHRWQIRN SPV</td>
</tr>
</tbody>
</table>

Table 21: Peptide sequences of phage clones selected with the anti-gp350 virus-neutralising MAb 72A1 from the 15 mer library. The amino acids in bold type are those which have been noted as being common to a number of different selected clones. Some clones are repeated as they show homology to 2 "groups".
<table>
<thead>
<tr>
<th>Group no.</th>
<th>Clone no.</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2522</td>
<td>S RRKSGIL G</td>
</tr>
<tr>
<td></td>
<td>2523</td>
<td>S RTKSDIL G</td>
</tr>
<tr>
<td>2</td>
<td>25212</td>
<td>S SSSTYRY G</td>
</tr>
<tr>
<td></td>
<td>25215</td>
<td>S SHPDTYR G</td>
</tr>
<tr>
<td></td>
<td>25219</td>
<td>S SHPITYR G</td>
</tr>
<tr>
<td>3</td>
<td>2525</td>
<td>S TRVDSSP G</td>
</tr>
<tr>
<td></td>
<td>2529</td>
<td>S TRVDSSH G</td>
</tr>
<tr>
<td></td>
<td>2526</td>
<td>S SPRVPR- G</td>
</tr>
<tr>
<td>4</td>
<td>25212</td>
<td>S SSSTYRY G</td>
</tr>
<tr>
<td></td>
<td>2526</td>
<td>S SPRVPR- G</td>
</tr>
<tr>
<td></td>
<td>25218</td>
<td>S SLWARHR G</td>
</tr>
<tr>
<td></td>
<td>2524</td>
<td>S SVMSVTT G</td>
</tr>
<tr>
<td></td>
<td>25215</td>
<td>S SHPDTYR G</td>
</tr>
<tr>
<td></td>
<td>25219</td>
<td>S SHPITYR G</td>
</tr>
<tr>
<td></td>
<td>2521</td>
<td>S SHQQSST G</td>
</tr>
<tr>
<td></td>
<td>25214</td>
<td>S SHLIQQQR G</td>
</tr>
<tr>
<td></td>
<td>2525</td>
<td>S TRVDSSP G</td>
</tr>
<tr>
<td></td>
<td>2529</td>
<td>S TRVDSSH G</td>
</tr>
<tr>
<td>5</td>
<td>25211</td>
<td>S VDLRRTAF G</td>
</tr>
<tr>
<td></td>
<td>25213</td>
<td>S TFLRFPL G</td>
</tr>
<tr>
<td>Ungrouped</td>
<td>25216</td>
<td>S AAPGTLPG</td>
</tr>
</tbody>
</table>

Table 22: Amino acid sequences displayed on phage clones selected with the anti-gp350 virus-neutralising MAb 72A1 from the 7 mer unconstrained library. The amino acids in bold type are those which have been noted as being common to a number of different selected clones. Some clones are repeated as they show homology to 2 'groups'
<table>
<thead>
<tr>
<th>Group no.</th>
<th>Clone no.</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C7CN</td>
<td>S C Y K S L P E Q C G</td>
</tr>
<tr>
<td></td>
<td>C7C18</td>
<td>S C H P W F Q T I C G</td>
</tr>
<tr>
<td></td>
<td>C7C4</td>
<td>S C S P A Q F L A C G</td>
</tr>
<tr>
<td></td>
<td>C7C5</td>
<td>S C N P N Q L Y L C G</td>
</tr>
<tr>
<td></td>
<td>C7CC</td>
<td>S C P T L L Q L H C G</td>
</tr>
<tr>
<td></td>
<td>C7C28</td>
<td>S C L G S G Q Y W C G</td>
</tr>
<tr>
<td>2</td>
<td>C7C10</td>
<td>S C T L Q K P W M C G</td>
</tr>
<tr>
<td></td>
<td>C7C14</td>
<td>S C H L K D P N K C G</td>
</tr>
<tr>
<td></td>
<td>C7C15</td>
<td>S C D R L S G P R C G</td>
</tr>
<tr>
<td></td>
<td>C7C27</td>
<td>S C F T E L P F S C G</td>
</tr>
<tr>
<td>Ungrouped</td>
<td>C7CB</td>
<td>S C I I N G P V N C G</td>
</tr>
<tr>
<td></td>
<td>C7C7</td>
<td>S C N T P T P R S C G</td>
</tr>
<tr>
<td></td>
<td>C7C9</td>
<td>S C T T T L N T S C G</td>
</tr>
<tr>
<td></td>
<td>C7C21</td>
<td>S C S K S S V R S C G</td>
</tr>
</tbody>
</table>

Table 23: Amino acid sequences displayed on phage clones selected with the anti-gp350 virus-neutralising Mab 72A1 from the 7 mer constrained library. The amino acids in bold type are those which have been noted as being common to a number of different selected clones.
### Table 24: Amino acid sequences displayed on phage clones selected with the anti-gp350 virus-neutralising MAb F2.1 from the 15 mer library.

The amino acids in bold type are those which have been noted as being common to a number of different selected clones.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Clone no.</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F21B4</td>
<td>GP RSHWFDTDPRDHR SPV</td>
</tr>
<tr>
<td></td>
<td>F21A3</td>
<td>GP KYVRRHWFGHGHKTS SPV</td>
</tr>
<tr>
<td>2</td>
<td>F21A2</td>
<td>GP KKRHLSHFYTQSVKV SPV</td>
</tr>
<tr>
<td></td>
<td>F21B1</td>
<td>GP SLAEVINFFSYGVRV SPV</td>
</tr>
<tr>
<td>3</td>
<td>F21B2</td>
<td>GP HCHRWSCREYRAQDR SPV</td>
</tr>
<tr>
<td></td>
<td>F21A4</td>
<td>GP HSFYIHALTGGSMHR SPV</td>
</tr>
</tbody>
</table>

The gp350/220 peptide sequence shows similarity with group three from the 15 mer library only, no other significant similarity between any of these sequences and the primary aa sequence of gp350 was found. Screening the library with a different monoclonal antibody selects clones expressing peptide sequences completely unrelated to those selected with 72A1. However, two identical sequences were found after three rounds of biopanning of the 15 mer library with 72A1 and F2.1, these are 1 13 and 1 15 in table 21, and F21A4 and F21B2 in table 24.

Both the 7 mer constrained and unconstrained libraries showed deletions within the insert or absence of an insert in almost 50% of the clones sequenced. As with the 12 mer library, this may have been due to non-specific selection of phage. In order to determine at what stage the selection of phage without inserts took place, a number of clones were selected from the primary libraries and from various stages throughout the biopanning procedure. The sequences of the selected clones are detailed in table 25 below.

---

Table 24: Amino acid sequences displayed on phage clones selected with the anti-gp350 virus-neutralising MAb F2.1 from the 15 mer library. The amino acids in bold type are those which have been noted as being common to a number of different selected clones.

The gp350/220 peptide sequence shows similarity with group three from the 15 mer library only, no other significant similarity between any of these sequences and the primary aa sequence of gp350 was found. Screening the library with a different monoclonal antibody selects clones expressing peptide sequences completely unrelated to those selected with 72A1. However, two identical sequences were found after three rounds of biopanning of the 15 mer library with 72A1 and F2.1, these are 1 13 and 1 15 in table 21, and F21A4 and F21B2 in table 24.

Both the 7 mer constrained and unconstrained libraries showed deletions within the insert or absence of an insert in almost 50% of the clones sequenced. As with the 12 mer library, this may have been due to non-specific selection of phage. In order to determine at what stage the selection of phage without inserts took place, a number of clones were selected from the primary libraries and from various stages throughout the biopanning procedure. The sequences of the selected clones are detailed in table 25 below.
<table>
<thead>
<tr>
<th>Library</th>
<th>Round no</th>
<th>Clone no.</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 mer</td>
<td>Unselected</td>
<td>1</td>
<td>SSLNP&lt;P&gt;NPG</td>
</tr>
<tr>
<td>7 mer</td>
<td>Unselected</td>
<td>2</td>
<td>SNPWAYYQG</td>
</tr>
<tr>
<td>C7C</td>
<td>Unselected</td>
<td>1</td>
<td>CSLV&lt;KLGAC</td>
</tr>
<tr>
<td>C7C</td>
<td>Unselected</td>
<td>2</td>
<td>CSTRSVVLC</td>
</tr>
<tr>
<td>C7C</td>
<td>Round 2</td>
<td>C7C1</td>
<td>no insert</td>
</tr>
<tr>
<td>C7C</td>
<td>Round 2</td>
<td>C7C2</td>
<td>CT&lt;TTAYQT&lt;NC</td>
</tr>
<tr>
<td>C7C</td>
<td>Round 2</td>
<td>C7C6</td>
<td>CAPFINRS&lt;NC</td>
</tr>
<tr>
<td>C7C</td>
<td>Round 2</td>
<td>C7C10</td>
<td>C&lt;NPANTDM&lt;NC</td>
</tr>
<tr>
<td>12 mer</td>
<td>Unselected</td>
<td>1</td>
<td>SSL&lt;ICVT&lt;VTVASAG</td>
</tr>
<tr>
<td>12 mer</td>
<td>Unselected</td>
<td>2</td>
<td>SFAANKMPFGPGSG</td>
</tr>
<tr>
<td>12 mer</td>
<td>Unselected</td>
<td>3</td>
<td>GSALVTEPSI&lt;AWG</td>
</tr>
<tr>
<td>12 mer</td>
<td>Unselected</td>
<td>4</td>
<td>SAVVVFYPSPNLAG</td>
</tr>
<tr>
<td>12 mer</td>
<td>Round 2</td>
<td>12M1</td>
<td>deletion mutant (a single base missing)</td>
</tr>
<tr>
<td>12 mer</td>
<td>Round 2</td>
<td>12M4</td>
<td>SHFNHSIRLHNAAG</td>
</tr>
<tr>
<td>12 mer</td>
<td>Round 2</td>
<td>12M5</td>
<td>STDLQIKRHLGHGG</td>
</tr>
<tr>
<td>12 mer</td>
<td>Round 3</td>
<td>12M2</td>
<td>no insert</td>
</tr>
<tr>
<td>12 mer</td>
<td>Round 3</td>
<td>12M3</td>
<td>no insert</td>
</tr>
<tr>
<td>12 mer</td>
<td>Round 3</td>
<td>12M6</td>
<td>deletion mutant (a single base missing)</td>
</tr>
</tbody>
</table>

Table 25: Amino acid sequences displayed on clones selected from the primary libraries and form various stages throughout the biopanning procedure.
All clones isolated from the un-panned libraries had complete inserts without any deletions, this suggested that selection of phage without inserts or with deletions within the inserts, occurred during the biopanning process. Four clones were selected from the 7 mer constrained library and six from the 12 mer library. These represented phage from 7 mer constrained library round two eluate, un-amplified and 12 mer library round two and round three eluate, un-amplified.

These findings indicate that the selection of clones without inserts and with mutations within the insert occurs before the final round of panning and with amplification of the eluate from each round, these clones are being amplified also. It may be that phage without inserts may have a growth advantage over those with an insert. If a small fraction of the phage population are without inserts after the first or second round of panning, these may outgrow phage with inserts during the amplification. However, deletions within the insert disrupts the ORF of the pIII gene, as this protein is involved in infection of host cells, it would be expected that these phage could not propagate during amplification. As all phage from the primary unpanned library were without mutation, it is likely that phage without inserts not expressing a peptide have a growth advantage.

**4.2.3.1 Comparison of Sequenced Clones**

*15 mer Library panned with 72A1 (Table 21)*

These clones were divided into three groups which had similarities in amino acid sequence, a fourth group was found which consisted of two sequences 100% identical to two sequences found after panning this library with the monoclonal antibody F2 1 (1 13 and 1 15 in table 21, and F21A4 and F21B2 in table 24), which is specific for the glycoprotein gp85. This may have occurred due to binding of phage to streptavidin or the plate or non-specific binding to this class of antibody (class IgG2a), these phage may then be released into the eluate. However, measures were taken to prevent this occurring (section 2.6) Clones 1 1 and 1 6 are allocated to two groups as their amino acid sequences fit the consensus sequences of both groups. Six of the 18 peptides isolated possessed no evident common pattern in their amino acid sequences.
The amino acids in bold type are those which feature in every peptide within that group, the other residues appear in at least 2 peptides within each group.

Peptides from group three had a number of amino acids which were common to a region close to the carboxy terminus of the gp350/220 protein, this was the only sequence found within the native protein which could be directly correlated with any of the selected peptides.

gp 350/220  
aa 862  L  V  L  Q  W  A  S  L  A  V  L  T  aa 873

As the epitope may be conformational (Morgan et al., 1984a), it is possible that a number of the amino acids which constitute the epitope are located in this region of the protein. The remainder of the amino acids which complete the epitope may be located at positions on the primary sequence which are distant from the carboxy terminus. When the protein is folded in its three-dimensional form these residues may then be in a close spatial arrangement.

7 mer Library panned with 72A1 (Table 22)

Of these 15 peptides, 5 groups were identified. All the peptides in groups 2 and 3 are also present in group 4. One clone was found which had no similarity to the other peptides that were sequenced.

Group 1  R  K  S  I  L

Group 2  S  H  P  T  Y  R

Group 3  T  R  V  D  S  S
In comparison with the amino acid sequence of gp350/220 there were no apparent similarities, however, the double serine motif which appears frequently in group 4 and also appears in group 3 can be seen at eight locations on the gp350/220 primary amino acid sequence, predominantly toward the carboxy terminal half of the protein.

**7 mer constrained Library panned with 72A1 (Table 23)**

The set of peptides selected from this library were divided into two specific groups. Four of the 14 clones sequenced had no particular similarity to the others in the set.

**Group 1**

\[ \ldots L/P \quad Q \quad L \ldots \]

**Group 2**

\[ \ldots L/K \quad P \ldots \]

As with the 7 mer library there were no significant homologies (2-3 aa) between these groups of amino acids and the native gp350/220 sequence could be found.

**15 mer Library panned with F2 1 (Table 24)**

Six clones were sequenced from the third eluate after panning the 15 mer library with F29 167. These sequences were compared with the peptides found when panning with 72A1 to determine if the panning procedure was isolating sequences which did not have particular specificity for the antibody used in the panning procedure. Two specific groups were noted which possessed a number of identical amino acids, and two peptides were found which had identical sequences to two of those selected by 72A1, these are shown in group 4 of table 22, and group 3 of table 24.

**Group 1**

\[ \ldots R \quad H \quad W \quad F \ldots \]

**Group 2**

\[ \ldots F \quad F \quad Y \quad V \quad V \]
In general there are differences in the relative positioning of the amino acids which are common, within each group, they may differ in space by 1-2 amino acids. The interaction between an antibody and its epitope is very precise and specific, however as these peptides are displayed freely on the surface of the phage via the pIII protein it may be possible for these peptides to adapt the required conformation in order to bind the antibody.
4.2.3.2 Sequence Alignment according to Amino Acid class

The sequences selected from each library were also compared in relation to the class of amino acid present as opposed to the individual amino acids, i.e. polar, charged +/-; polar, uncharged; nonpolar. These alignments are shown in figures 53-56, for each figure the following colours denote the different classes of aa:

- **S T N Q Y C** polar, uncharged
- **K R H** polar, + charge
- **D E** polar, - charge
- **G A V L I M P F W** Nonpolar

![Alignment of the sequences selected from the 15 mer peptide library using 72A1, according to amino acid class.](image)

Figure 53: Alignment of the sequences selected from the 15 mer peptide library using 72A1, according to amino acid class.

Comparison of the sequences according to the class of amino acid allows comparison of all the selected sequences without division into smaller groups. Figure 53 shows a definite pattern in the majority of the sequences, there is a core of nonpolar amino acids
flanked by one or more polar positively charged amino acids, alternating with polar, uncharged amino acids in many cases.

Figure 54: Alignment of the sequences selected from the 7 mer peptide library using 72A1, according to amino acid class.

Comparison of the sequences selected from the 7 mer library (by 72A1) by this method also shows a definite pattern in the aa arrangement between all the sequences (figure 54). The pattern does not compare closely to that of the 15 mer sequences, however there is consistently the presence of a polar, uncharged aa followed by a positively charged, polar aa.
Figure 55: Alignment of the sequences selected from the 7 mer constrained peptide library using 72A1, according to amino acid class.

The pattern observed with the 7 mer constrained sequences (figure 55) shows no similarity with those of the 15 mer and 7 mer unconstrained sequences (figures 53 and 54). This may be due to the fact that these peptides are constrained and thus bind the antibody in a different way to the linear peptides.

Figure 56: Alignment of the sequences selected from the 15 mer peptide library using F2.1, according to amino acid class.

The sequences selected from the 15 mer library by F2.1 (figure 56) do not show any consistent pattern, however, had a greater number of clones been analysed, a specific pattern of aa.s might have begun to emerge. There is no obvious relationship with those selected using 72A1 from any of the other libraries.
Figure 57 shows the primary amino acid sequence of gp350, with the amino acids coded according to amino acid class using the same colour code previously described.

![The primary amino acid sequence of gp350 colour coded for amino acid class.](image)

According to this method of comparison there was no significant similarity between the selected sequences from any of the libraries and the primary gp350 sequence. If the epitope is conformational or has a sugar component this would be expected.

### 4.2.4 Deglycosylation of gp350 and Western blotting

Western blot analysis was performed using two of the monoclonal antibodies, 72A1 and F29167, to aid the determination of the type of epitope recognised by each antibody. Both purified gp350 (a gift from Dr. A. Morgan, University of Bristol, purified by affinity selection from cell culture). Figure 58 shows a 7.5% SDS PAGE protein gel of the gp350 protein. The protein was blotted as described in section 2.4.8, and probed with both 72A1 and F29.167. As can be seen in figure 59 (western blot) both antibodies bound to the purified gp350 molecule (500ng) in its fully glycosylated form. There were two high molecular weight bands, gp350 and possibly a gp350 aggregate, however with absence of appropriate size markers it is not possible to confirm this. The higher band was not visible when analysed on SDS-PAGE (figure 58), but could be seen on western
blot (figure 59) This result confirms that both monoclonal antibodies recognise the glycosylated form of the protein. Conjugate only did not bind (data not shown).

It has previously been shown that 72A1 does not interact with gp350/220 in a denatured state, indicating that the epitope is most likely discontinuous (Morgan et al., 1984a). However, peptide reaction in immunoblots may also be achieved by non-linear epitopes where the reacting portions are in relatively close proximity (Fack et al., 1997). This may explain this interaction of 72A1 with gp350/220 as can be seen in figure 59, lanes 2 and 3.

![Figure 58: A 7.5% SDS PAGE gel of purified gp350. Lanes are as follows, 1- Protein markers, 2- gp350 (50 ng), 3- gp350 (250 ng), 4- gp350 (500 ng).](image-url)
Figure 59: Western blot analysis of purified gp350, probed with F29.167 (lane 1) and 72A1 (lanes 2 and 3) from a 7.5% SDS PAGE. Lanes 1-3 gp350 (500 ng), lane 4 - Protein markers

A sample of purified gp350 was deglycosylated in order to determine if the carbohydrate (CHO) moiety is required for antibody recognition, the sugars may form part of the epitope or may be required structurally for binding of the monoclonal antibodies to an epitope on the amino acid sequence. The carbohydrate moiety of gp350/220 is known to be made up of both O-linked and N-linked types and to constitute about 50% of the molecular mass. This high carbohydrate content of gp350/220 appears to confer resistance to proteolysis (Morgan et al., 1984).

A cocktail of the deglycosylation enzymes, N-glycosidase F, O-glycosidase, and Neuraminidase was used. N-glycosidase F cleaves all types of asparagine bound N-glycans provided that the amino-group as well as the carboxyl-group are present in a peptide linkage and that the oligosaccharide has the minimum length of the chitobiose core (Data sheet - Boehringer Mannheim). O-glycosidase liberates the disaccharide Gal b(1-3) GalNAc from O-glycans, which is bound to serine or threonine as a core unit (Data sheet - Boehringer Mannheim). Neuraminidase cleaves terminal sialic acid residues which are α2,3-, α2,6- or α2,8- linked to Gal, GlcNAc, GalNAc, AcNeu, GlcNeu, oligosaccharides, glycolipids or glycoproteins (Boehringer Mannheim catalogue, 1998). The protein had been purified from cell culture supernatant, but its concentration was
had not been determined. A 100 \( \mu l \) volume was denatured and a 30 \( \mu l \) sample was used for deglycosylation. As a negative control, 30 \( \mu l \) of the denatured protein was incubated as for deglycosylation without the deglycosylation enzymes (all other reagents were included), see section 2.5.10.

Both the glycosylated and deglycosylated forms were analysed on a 6\% SDS-PAGE, and blotted onto nitrocellulose as described in section 2.4.8 and 10 \( \mu l \) of each deglycosylation reaction was run on the gel. The blot was probed with F29 167 and MDP 61 1 4, a polyclonal anti-gp350/220 preparation from a rabbit (supplied by Dr A Morgan), all were diluted 1/1000 in blotto. As can be seen in figure 60, the polyclonal antibody preparation recognises both the glycosylated and deglycosylated forms of the glycoprotein. The main band in figure 60, lane 1, of the deglycosylated gp350 appears at approximately 130 kD, as would be expected (Morgan et al., 1984). It is possible that small indigestible traces of CHO remain and contribute to antibody-binding. F29 167 reacts with the deglycosylated form, but not with the glycosylated form in this particular blot, possibly due to a low protein concentration. This was not due to gp350 degradation as it wasn’t degraded under similar conditions i.e. lane 2 shows the same material as lane 4, figure 60, it may have been a localised problem with protein transfer on this region of the filter during blotting. However, as can be seen in figure 59, F29 167 does recognise the glycosylated form of gp350. These results suggest that the epitope recognised by F29 167 is linear and that the carbohydrate moiety is not required for recognition nor does it mask the epitope. This experiment was not performed with 72A1, although this would have been desirable. Unfortunately, this antibody was no longer available at that time. The MAb F2 1 reacts with gp85 which was not available.
Glycosylated

Deglycosylated

Figure 60: Western blot analysis of gp350/220, glycosylated and deglycosylated, probed with F29.167 and MDP 61.1.4 antibody preparations. The lanes are as follows, 1- gp350/220 deglycosylated, 2- gp35/220 glycosylated, 3- gp350/220 deglycosylated, 4- gp350/220 glycosylated, 5- Protein markers. Lanes 1&2 were probed with the polyclonal antibody preparation MDP 6114, Lanes 3&4 were probed with F29 167.

4.2.5 Screening of Selected Phage Clones

A number of procedures were carried out to identify selected phage from the third round eluate, which displayed peptides which bound specifically to the monoclonal antibody used in the biopanning procedure, they are now described in turn.

4.2.5.1 Colony Lifts and Plaque Lifts

The absence of cell killing in fd-tet and its derivatives, including fUSE5 used here (15 mer library), is probably due to a defect in minus-strand synthesis (Smith, 1988) This defect allows titering of this library as transducing units (TU) in the appropriate bacterial strain, in this instance Escherichia coli K91Kan Each colony or TU represents a single successful infection This method of titering is more appropriate for this vector as titering as plaque forming units (pfu) is not practical due to the very tiny plaques which are formed The 7 mer, 12 mer, and 7 mer constrained libraries could all be titered as pfus
These methods enable one to screen larger numbers of plaques and colonies. The colony lifts and plaque lifts involved a format which was completely different from that which took place during biopanning, the phage were immobilised on nitrocellulose in each case as opposed to plastic. Both colony lifts and plaque lifts were performed as described in section 2.6.8, colony lifts of TUs for the 15 mer library and plaque lifts of pfu's for the M13 based libraries. The screening was performed on titers of the eluate from the third round of panning, before amplification. Positive and negative controls were dotted onto nitrocellulose and incubated as for the lifts themselves (See table 26 for details of the control reagents).

<table>
<thead>
<tr>
<th>Control</th>
<th>Concentration</th>
<th>Volume (μl)</th>
<th>Reaction (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse-AP Antibody</td>
<td>neat</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Anti-mouse-POD</td>
<td>neat</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>72A1</td>
<td>1mg/ml</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>gp350</td>
<td>50 μg/ml</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>BSA</td>
<td>100 μg/ml</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 26: The Controls used for the Colony/Plaque Lifts. (see figures 61A and 61B)
Figure 61A: Plaque lift of pfu.s from the eluate of the third round of panning with the 7 mer constrained library using the alkaline phosphatase anti-mouse conjugate and BCIP/NBT substrate. The plate used was a $10^{-1}$ dilution of the eluate with 30-300 plaques.

Figure 61B: The controls used for the plaque lift. Column 1- Anti-mouse-AP conjugate (2 μl neat), column 2- 72A1 (2 μg), column 3- gp350 (100ng), column 4- BSA (200ng).
Figure 62A: Plaque lift of pfu.s from the eluate of the third round of panning with the 7 mer constrained library using the peroxidase anti-mouse conjugate and a chemiluminescent substrate. The plate used was a $10^3$ dilution of the eluate with 30-300 plaques.

Figure 62B: The controls used for the plaque lift. Row 1- Anti-mouse-POD conjugate, 200 µl (1/1000, 1/500, 1/100), row 2- 72A1 (2 ng, 10 ng, 20 ng, 200 ng, 2 µg), row 3- gp350 (2 ng, 10 ng, 100 ng, 1 µg), row 4 - BSA (10 ng, 20 ng, 2 µg)
As can be seen from figures 61A-62B the controls reacted as expected, however no positive clones were visible on the plaque/colony lifts. No positive clones were found using the alkaline phosphatase conjugate and BCIP/NBT as substrate, therefore a chemiluminescence-based detection system was used (Supersignal, see section 2.1.4) for greater sensitivity. However, even with increased sensitivity there were no positive clones identified. Initially it was reasoned that this might have been due to a low concentration of phage in each plaque. If the peptides displayed were of low affinity and at a low concentration then it might be possible that this method for detection would not be sensitive enough to detect such peptides. Because the peptide is displayed on the pIII protein there are only 5 copies per phage particle, this method might be more suitable for phage displaying the peptide on the pVIII protein as there are ~2700 copies of this protein on each virion. Therefore, even low affinity peptides could be detected due to their abundance. A DOT blot procedure was then considered as a means of detecting positive clones, as it would be possible to screen at a higher phage concentration, thus allowing the detection of positive clones which may have a low affinity for 72A1.

4.2.5.2 DOT Blotting

A number of phage clones were screened using the same phage purification procedure as that designed for analysis by ELISA (section 2.5.4 and section 2.6.6). This was done in order to sufficiently concentrate the number of virions thus possibly enabling the detection of clones positive for 72A1 binding peptides using a DOT blot. The phage were titered before using in the DOT blot, to determine the concentration of pfu s. The following table gives an example of the concentration of phage used in the DOT blot experiment.

218
Table 27: Titering results of a selection of phage purified from the eluate from the third round of panning of the 7 mer library with 72A1.

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Titer (*10⁹ pfu/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2522</td>
<td>3.6</td>
</tr>
<tr>
<td>2523</td>
<td>4.5</td>
</tr>
<tr>
<td>25212</td>
<td>12.2</td>
</tr>
<tr>
<td>25215</td>
<td>12.8</td>
</tr>
<tr>
<td>25219</td>
<td>5.6</td>
</tr>
</tbody>
</table>

The procedure was carried out as detailed in section 2.5.9. Figure 63 shows in diagramatic form, the format of the assay. A chemiluminescence-based detection system was used in order to achieve a higher level of sensitivity.

Figure 63: DOT blot format for detection of 72A1 - specific peptides on phage particles selected during biopanning.

The same controls were used as for the plaque lifts, i.e. BSA, gp350, 72A1 and anti-mouse peroxidase conjugate, at a range of concentrations. Phage purified from the primary, un-panned libraries, were also used as negative controls. This method of screening was used for both the 7 mer and 7 mer constrained library. As with the plaque/colony lifts all the positive controls showed a very strong signal (indicating the high sensitivity of this method) and all negative controls were negative. Figure 64 shows
a photograph of the auto-radiograph developed after 30 s exposure to the nitrocellulose filter. Five clones from the 7 mer constrained library were spotted in triplicate on nitrocellulose using a dot-blotting apparatus which was connected to a vacuum pump.

Figure 64: Auto-radiograph of DOT Blot assay carried out with phage from the 7 mer constrained library. Rows are as follows, 1- gp350 (100 ng), 2- 72A1 (2*20 ng, 10 ng, 1 ng), 3- Anti-mouse-POD conjugate (3*1/1000, 1/500), 4- BSA (50 ng, 20 ng), 5- clones 31,32,33,34, 6- clones 35,36,A,B, 7- clones 37,38,39,310, 8- clones 311,312,C,D (A-D are clones selected from the primary, un-panned library)

No positive clones were identified. It was thought that as the method of immobilisation of phage was different to that used during biopanning, that detection by the monoclonal antibody binding may be somewhat diminished.

4.2.5.3 BIAcore Analysis

It was thought that lack of sensitivity might also be a contributory factor in the failure to isolate positive clones. BIAcore analysis was then considered, as this technology represents one of the most sensitive antibody-antigen assay techniques available.
technology involves immobilisation of the target molecule, i.e. 72A1, on a sensor chip, and then passing a preparation of phage library over this chip. The interaction is measured as a change in response units (RU), which is caused by change in the refractive index of the chip surface due to the increase in mass on the surface of the chip (Jonsson and Malmqvist, 1992). A very low signal (243.9 RU) was obtained when assaying the interaction between 72A1 and purified gp350. (Figures 65 and 66 show the sensorgrams obtained in the control reaction, i.e. initial binding of 72A1 to the sensor chip followed by binding of gp350.) A typical response for \(10^{13}\) pfu/ml, from a third round eluate, reacted with the target molecule is 1870 RU (Lasonder et al., 1994). Phage would be expected to have a much lower signal than that of gp350 therefore a higher concentration of phage would be required. In conclusion, BIAcore analysis was not able to show satisfactory Ab-Ag interaction under the conditions used. As not enough MAb or purified gp350 was available for optimisation of the procedure, it was decided not to proceed with analysis of phage clones by this method.

Figure 65: Sensorgram of immobilisation of 72A1 on the sensor chip. Between 1750 s and 2250 s the change in response units is \(1000\) RU, this represents immobilisation of the antibody.
Figure 66: Sensorgram of the binding of gp350. The change in RU of 243 9 RU at ~500 s represents binding of gp350.

4.2.5.4 ELISA Analysis

As all of these methods of screening use a different format to that used in the biopanning procedure it was decided to try an ELISA format which would be more similar to the method of panning, which, in the case of the 15 mer and 7 mer libraries, involved prior immobilisation of the target antibody on plastic followed by the addition of free phage. The plaque/colony lifts and DOT blot method required immobilisation of the phage onto nitocellulose, which does not occur during solution biopanning and which may have resulted in a difference in the accessibility/binding characteristics of the displayed peptide with respect to the target antibody 72A1. A number of different ELISA formats were considered, see figure 67 (A-C), for a schematic representation of these assays.
Figure 67: Possible ELISA formats for detection of 72A1 - specific peptides on phage particles selected during biopanning.
The first method considered (A) involves direct immobilisation of the phage on the 96 well plate which may result in uneven coating of the different clones, using a monoclonal antibody to immobilise the phage would give more uniform coating of the phage particles as in diagram (B). However, to follow the biopanning procedure more closely it was decided to coat the plate with 72A1, and follow this with incubation with the purified phage clones. A rabbit anti-phage antibody was then used, followed by an anti-rabbit alkaline phosphatase conjugate, and pNPP as substrate. Phage selected by F29 167 and randomly chosen phage that were purified from the primary library (without panning) were used as negative controls.

ELISA results showed a positive reaction between phage purified from the unselected library as well as those selected during biopanning. There was no direct interaction between 72A1 and either the anti-phage antibody or the anti-rabbit alkaline phosphatase antibody (this was indicated by the absence of signal). When phage were present from the un-panned library or from the third round of panning with 72A1 there was always a positive result. Increasing the stringency of the washes (with 0.5% Tween 20) between the incubation periods did not appear to reduce this interaction.

As this procedure replicates on a smaller scale the interactions which take place during biopanning, it highlights the possibility that this monoclonal antibody (72A1) may be capable of binding filamentous phage at some site other than specifically with the surface displayed peptide. As a result the antibody binds phage from a number of different sources which do not necessarily display a peptide which mimics its epitope. However, this still does not explain the fact that related phage clones were obtained. This binding was not seen in DOT blotting or in colony/plaque lifts. In the case of the plaque/colony lifts this may be due to low concentration of phage particles. In DOT blotting it may be that as the format is different and involves direct immobilisation of the phage on nitrocellulose followed by probing with 72A1, that the accessibility of the particular binding site is reduced.
4.3 Discussion

4.3.1 Epitope mapping of gp350 and the neutralising Mab 72A1

The monoclonal antibody 72A1 neutralises both B95-8 and P3HR-1 indicating that the epitope is present in both type A and type B viruses (Sairenji et al., 1988), this epitope was thought to be located within the amino-terminal 162 aa (Tanner et al., 1988). However, there appears to be species-specific variation in the immune response to EBV, as the epitopes found using serum from healthy seropositive humans were located toward the carboxy terminus of gp350 and were thought to be discontinuous in nature (Pither et al., 1992a), while those found after immunisation of rabbits were amino-terminal and continuous (Pither et al., 1992b). This difference in response may also depend on whether the individual is naturally infected or vaccinated, also, the molecule's host cell-specific pattern of glycosylation will influence the accessibility and structure of the gp350/220 molecule. It has been shown that 72A1 binds gp350 on western blot of a denaturing SDS-PAGE (section 4.2.2, figure 59), therefore it may be that the reacting portions of a discontinuous epitope are in relatively close proximity and are capable of reaction in immunoblot (Fack et al., 1997).

4.3.2 Sequence Comparisons

As shown in section 4.2.3.1, there are a number of defined groups within each set of sequenced clones for each particular library, divided on the basis of sequence similarity. Within the 15 mer library the three defined groups showed many apparent sequence comparisons with up to six of the fifteen amino acids conserved between two sequences within each group. This indicates that there was some level of affinity selection. In the case of the 7 mer library, groups one to three showed between five and six conserved residues between two sequences within each group. Due to the high number of deletion mutants and clones without inserts which were found, there were a limited number of sequences for comparison, however, affinity selection does appear to have occurred. As can be seen in section 4.2.3, sequencing albeit a small number of clones from either the primary library or earlier rounds of panning showed no relationship between the sequences of each clone. If there were no affinity selection after three rounds of panning this would also have been the case for the clones sequenced after round three. Clones...
selected from the 7 mer constrained library show a lower degree of sequence homology, with only up to three conserved residues.

Comparison of the peptides according to amino acid class, as in section 4 2 3 2 shows a more defined pattern for the sequences selected, by 72A1, from both the 15 mer and 7 mer linear peptide libraries (figures 21 and 22) The type of amino acid is important in defining an epitope or mimotope, although the actual aa's may not be identical, if aa's of the same type are present in similar positions the same interaction between the peptide and antibody may occur In the case of the 15 mer sequences there is a core of nonpolar aa's in the majority of the peptides, preceded by at least one polar, positively charged aa, in many cases a polar uncharged aa is present between two of the positively charged aa's. Five of the 15 peptides selected from the 7 mer library are similar in that they too have a polar, uncharged amino acid, followed by a positively charged residue and then a nonpolar residue. However, there was no obvious relationship between the primary amino acid sequence of gp350 and the selected phage clones by this method of comparison. This reinforces the possibility that the epitope is conformational. In general, alignment by this method allows a less rigid means of sequence comparison, which may reflect more clearly the type of selection permitted by phage display technology.

Amino acid sequence patterns may be very obvious or often very obscure, and the type of library screened may play a crucial role in identification of some binding domains as may the type of interactive domain itself. In some cases the consensus sequence is evident in 100% of clones, however it is also possible that despite persistent effort and varied strategies that a consensus sequence may not be obtained (Burritt et al., 1995). It is usually desirable to select several similar but unique clones which support a consensus amino acid sequence. It is frequently the case that the conserved residues of the phage sequences are specifically spaced and may demonstrate which, if any, conservative substitutions can exist in these positions. There may also be a number of positions in which almost any residue appears, possibly indicating amino acids in which the side chain faces away from the interactive surface of the molecule. The small number of conserved residues found in the inserts selected from the constrained library may reflect this type of situation. As the inserts are constrained and have restricted conformational freedom, it is possible that only a small number of residues are required for high affinity binding to the
antibody. The other positions may therefore be redundant in antibody binding and thus allow for the presence of almost any amino acid.

As only one group of clones from the set of 15 mer sequences bears any similarity to the native gp350/220 primary amino acid sequence, it is likely the epitope is conformational, or that all other selected peptides are mimotopes of a linear epitope.

4.3.3 Methods of Panning

As the interaction between any antibody and its particular epitope is so specific, different biopanning methods and a panel of libraries were used in order to maximise the possibility of isolating the specific epitope or mimotope. The way in which the target molecule, in this case 72A1, is presented to the phage may also be critical to the selection of phage displaying peptides with high affinity for that molecule.

The high-affinity biotin-streptavidin interaction was exploited when panning the 15 mer library. This method was chosen in order to increase yields and reduce the requirement for antibody, compared to direct immobilisation of antibody on polystyrene. However, there may have been losses during the concentration procedure required prior to biotinylation and the extent of biotinylation of the antibody was not assayed. Due to these factors, the level of antibody immobilisation may not have been optimal. The fact that there were two identical sequences found after panning with two different antibodies 72A1 and F21, may indicate a non-specific interaction between the phage and streptavidin. A subtraction step was carried out in further biopanning, directly after the first round in order to remove these non-specific phage clones. This involved incubation of the eluate with a streptavidin coated plate and subsequent removal of the eluate followed directly by amplification. These factors may influence the type of peptides selected, using this particular method.

Bivalent binding of phage to the antibody may also occur using the biotin-streptavidin immobilisation method, or using direct immobilisation (for the 7 mer library). One antibody may bind more than one peptide on a particular virion or it may bind peptides from two different phage clones, and this bivalent binding may be irreversible. Therefore, affinity panning may not be able to distinguish between moderately high affinity peptides.
and very high affinity peptides (Scott and Smith, 1990) As a result low affinity binders may have bound the monoclonal antibody, 72A1 On screening selected clones by colony/plaque lifts or DOT blot, the absence of any visible interaction with 72A1 may be explained by the low affinity of these peptides

Limiting phage concentration in rounds two and three of biopanning was carried out in order to increase the number of high affinity peptides selected, however this also results in competition between phage for antibody binding. If lower affinity binders were preferentially selected in round one (due to bivalent binding, and the availability of excess antibody) and were in much greater excess than those with higher affinity, such a limitation on available antibody may have resulted in further reduction in the number of higher affinity clones selected and amplified in subsequent rounds of panning.

Both the 12 mer and 7 mer constrained library were panned via the solution binding procedure, using Protein G agarose beads. This method was chosen as it requires substantially less antibody than surface panning, can improve accessibility of the antigen binding site to phage-displayed peptides and can avoid potential partial denaturation of the antibody on the plastic surface. These beads have pores that are large compared to ligate but small compared to the long dimension of the virion, therefore it is likely that only a fraction of the immobilised ligate is available to the virions. The virions are probably thin enough to penetrate the pores end first and could bind a ligate inside the interior and become trapped very close to the ligate, thus increasing the chances of binding between peptides and antibody molecules. However, this may also result in the selection of phage with very low affinity peptides, as in this case it is the physical proximity of phage and antibody which enables binding as opposed to the presence of a peptide with a high affinity for the particular antibody.

Each method has advantages and disadvantages which influence the type of peptides selected. The use of small impermeable beads, such as polyacrylic oxirane beads may be a more suitable method for the selection of high affinity peptides. This method provides the advantages of increased surface area to volume ratio, reduced antibody usage and reduction in antibody denaturation, without the problem created by the porous nature of agarose. These beads have been successfully used in previous work by Bass et al., 1990.
It appears that the method which proves successful in the identification of a particular epitope or ligand is very much dictated by the nature of the specific protein-protein interaction.

### 4.3.4 Methods of Screening

Four different screening methods were used in order to maximise the chances that the clones selected had specific affinity for the antibody 72A1, and that they could specifically bind the antibody under alternative conditions. BIAcore analysis was abandoned due to the poor signal obtained using gp350 as positive control. None of the four methods proved successful in definitive identification of 72A1 specific peptides. In the case of the plaque/colony lifts this may have been due to the fact that, although more clones could be screened the phage were not at a high concentration, in addition if the displayed peptides are not of very high affinity it would be difficult to detect them by this method.

Using a DOT blot or ELISA format allowed concentration of the phage, thus increasing the possibility of detecting phage despite the probability that they displayed low affinity peptides. However, as with the plaque/colony lifts, DOT blotting of limited numbers of phage did not result in any positive clones. This might have been due to the differences, in antibody presentation to the phage. As discussed in section 4.3.3, the method of antigen presentation can be of great importance in selection of high affinity peptides, similarly, in the post-panning screening, antibody presentation may be of equal significance. Therefore, ELISA assay, which had the most closely related format, would be the likely method to give a reflection of the level of interaction between the selected phage and the monoclonal antibody.

Using this method of screening, 72A1 was seen to interact with all phage clones, including those from the primary un-panned library. This finding would indicate that the phage were binding the antibody by some means other than via the displayed peptide. If this were the case the significance of the sequence comparisons found would be highly questionable. It may be possible that there existed a combination of phage-antibody interactions, which involved the displayed peptide in some cases and not in others. This may also explain the selection of such a large number of phage without peptide inserts. If
these phage were capable of binding the antibody by some means other than via the displayed peptide, and these clones may have had a growth advantage due to the lack of an insert (possibly making them more infectious) and are preferentially propagated, this may be the reason that these clones constitute such a high percentage of the phage selected. These results reinforce the importance of the use of a range of different screening methods in conjunction with a range of alternative panning procedures and different libraries, for any particular ligand.

4.3.5 Screening Different Libraries

The screening of a number of different libraries is important in order to maximise the possibility of selecting the phage clone displaying the peptide or protein with the highest affinity for the ligate in question. The four main variables in any library are:

- phage vector
- fusion protein, pIII/pVIII
- peptide length
- choice of invariant flanking residues.

Manipulation of these variables will determine the success of ligate selection in conjunction with the chosen panning method. As discussed previously, the identification of short linear epitopes can be achieved using 6 to 15 mer linear peptide libraries, however the search for conformational or discontinuous epitopes generally requires longer inserts which are themselves constrained. The 12 mer library and 15 mer library were thought to be of sufficient length in order to select a discontinuous epitope/mimotope, however it is not only the length of the peptide which is significant. The library must encompass all the possible combinations of amino acid sequence in sufficient numbers. A 15 mer library involving all 20 amino acids has the potential to display \(3.3 \times 10^{19}\) \((20^{15})\) unique sequences. One of the primary limiting factors in the production of such a complex library is the efficiency of electrotansfection of host bacteria with recombinant phage DNA, therefore it is not possible to generate a library with this complexity. In the case of the 15 mer library used here, the complexity was \(1.1 \times 10^{12}\), considerably less than the number of possible sequences. It is possible that the sequence with the greatest affinity for 72A1 is not present in this library. The same may
be the case for the other linear peptide libraries which were screened, however, all combinations are not required

Binding domains may contain a relatively small number of anchor residues along longer peptide regions, identification of this type of epitope may depend more on probing libraries of longer peptides than those with the greatest diversity Libraries have been produced with up to 38 residues which are more diverse than libraries with the same number of phages bearing shorter peptides Peptides of this length as well as being more chemically diverse, can assume a variety of secondary structural motifs, such as α-helices, β-turns, and mimics of antigenic loops on the surface of different proteins Also, peptides of this length allow greater diversity in the regions flanking binding residues which could lead to improved affinity based on mechanisms which are dependent on secondary interactions between flanking residues and the target If these types of secondary interactions are important for the monoclonal antibody 72A1, the peptides of each of the linear libraries used may have been too short to select the epitope

The conformation assumed by longer peptides is dependent on the free energies of their amino acid sequences in the suspending medium This conformation may be quite different from that of the same amino acid sequence as it exists in the native form or bound forms of a protein, therefore, selection of certain binding motifs may only be possible when using libraries displaying peptides that assume specific conformation The cyclic form of the peptides in a constrained library should have restricted conformational freedom thus providing higher affinity and specificity for a receptor The more tightly a peptide segment is constrained, the less likely it is to bind any particular target, if it does bind, this binding is likely to be tighter and more specific It has also been found that phage from constrained peptide libraries are more effective as immunogens as they are more stable, and have a longer plasma half life than those from unconstrained peptide libraries (Ladner, 1995) One constrained library was screened, which had just seven residues, this peptide length may also have been too short in order to identify the epitope of the 72A1 antibody A constrained library with a longer insert would be more ideal, but didn’t exist at the time of this research
Screening of a gene-fragment library may prove to be the most successful method of isolating the epitope or mimotope of 72A1. Construction of such a library would involve DNasel digestion of plasmid DNA encoding the gp350/220 gene, and cloning of the resulting fragments into an appropriate phage vector such as fUSE5, as described by Fack et al., 1997. As the 15 mer library was the longest peptide library we screened, using a gene-fragment library would increase the possibility of recognising the 72A1 epitope if it is greater than 15 amino acids in length. Also, this type of library would encompass a much greater diversity of secondary structures which may also be crucial to the identification of this epitope. It has been shown using a gene-fragment library, that a single round of panning is sufficient to determine an epitope (Fack et al., 1997), this is possibly due to the fact that the library is several orders of magnitude smaller than a random peptide library. With just one round of panning required, the procedure is much simplified as the library is specific for a particular antigen.

4.3.6 Phage Display Technology

Screening of a wider range of libraries may not be successful in determining the epitope of 72A1 or identifying a mimotope. However, the results presented indicate that this technology does show affinity selection of peptides which may be reactive with 72A1. The interaction between the selected peptides and the monoclonal antibody is very dependent on the physical and chemical conditions in which these entities are presented to each other. As all antibody-antigen interactions are highly specific, a number of variables must be manipulated in order to find the conditions which most successfully replicate this interaction in vivo. In vitro interactions can vary considerably from those occurring in the body and it may not be possible to reproduce the necessary conditions for a particular protein-protein interaction, however 72A1 was shown to interact with gp350 on western blot.

The type of library screened appears to be the most influential factor in the selection of a specific ligand. In the case of epitope mapping, some knowledge of the type of epitope involved, aids the selection of a particular type of library or libraries to be screened. If the epitope is linear it is more likely to be identified using an unconstrained peptide library than if the epitope is known to be conformational. To date very little success has been had in the identification of discontinuous epitopes from linear peptide libraries. In
these cases constrained libraries with longer amino acid sequences, or gene-fragment libraries have proven to have more potential in epitope identification. Ideally, to have peptides of different lengths, that are constrained in several different frameworks of varying rigidity, would give the best opportunity of isolating the most specific, high affinity sequence. Construction of an antigen-specific library and the use of non-permeable beads for antibody immobilisation, may be the most effective way to identify the 72A1 epitope using phage display technology.
Chapter 5
Conclusions
5.1 Expression of recombinant EBV antigens in *E. coli*

Three recombinant EBV antigens, p18VCA, EA-D and truncated EBNA1 were expressed by the thiofusion expression system in *E. coli*, and were affinity purified using PAO. These have proven to be effective reagents for the identification of IM+ sera and sera from normal healthy individuals. The majority of serum samples, normal (seropositive), 'VCA+/-', 'EBNA+/-' and 'IM+', showed antibody responses similar to those expected, according to serological profiles determined by other methods (IFA and ELISA using cell culture-derived antigens). An IgM response to recombinant p18VCA, in conjunction with a rEBNA1 IgG negative response, showed greatest correlation with previously characterised 'IM+' sera. Eighty four percent of 'IM+' sera were also 'IM+' by this method, none of the normal sera were 'IM+', 68% were EBV seropositive. Of the 'VCA+' sera, 78% were also VCA IgM-positive by this method and 70% of 'EBNA+' were EBNA1-positive. In the case of sera that were characterised as being from individuals with primary CMV infection, 29% appeared to cross-react with rVCAp18, although this would need to be investigated further.

The presence of a strong background signal due to non-specific interactions with native *E. coli* proteins, may cause problems in effective diagnosis. There was no reactivity observed when western blotting was carried out with thioredoxin and a number of 'IM+' sera, therefore it does not appear to be necessary to remove the thioredoxin moiety using enterokinase. Also increasing concentrations of free thioredoxin were added to serum samples analysed by ELISA, with no reduction in the ELISA signal (data not shown). This would indicate the absence of any thioredoxin-specific antibody response. Affinity purification with PAO agarose effectively removed the majority of cellular *E. coli* proteins, however there was also substantial loss of recombinant fusion protein.

The results obtained show the potential of these recombinant EBV antigens for use as immunodiagnostic reagents for EBV-related disease.
5.2 Identification of anti-gp350 MAb-binding peptides

Comparison of the peptides selected from each of the RPLs by the EBV neutralising antibody 72A1, shows similarity in both amino acid content and the class of amino acids which predominate in the peptides. Particularly those selected from the 15 mer and 7 mer unconstrained libraries. Alignment by comparison of aa class (i.e. polar-charged, polar-uncharged and non-polar) allowed a less rigid means of sequence comparison, which may reflect more clearly the type of selection permitted by phage display of peptides less than 15 aas long. The small number of conserved residues found in the inserts selected from the constrained library may reflect the possibility that only a small number of residues are required for binding to the antibody. The other positions may therefore be redundant in antibody binding and thus allow for the presence of almost any amino acid, although some classes of aa may interfere with binding and would not be selected. This might be the case when one looks at the predominance of nonpolar amino acids following the polar charged amino acids in the peptides selected form the 15 mer library (Section 4.2.4.2, figure 53). The failure to demonstrate binding of 72A1 to any of the phage selected most likely indicates that the binding is (i) very low, or (ii) non-specific.

There was no apparent similarity between the primary amino acid sequence of gp350 and the amino acid sequences of the selected clones when compared with respect to amino acid class. This would be expected if the epitope were conformational in nature. However, it may not be possible to identify the epitope or mimotopes of 72A1 through the use of phage display technology. There are limitations, as with any scientific method, to the successful application of this technology. Specifically the identification of conformational epitopes has proven more elusive, than that of linear epitopes.

The identification of the neutralising epitopes on the gp350/220 molecule, and the subsequent synthetic production of these peptides could provide the basis of a subunit vaccine. A number of peptides could be incorporated in order to induce a broad immunological response, T cell epitopes could also be included in this type of vaccine, possibly as part of immunostimulating complexes (ISCOMS) (Morein et al., 1984). Recombinant phage displaying immunogenic peptides may also be directly considered as cell-free vaccine candidates (Dulbecco 1982; Folgori et al., 1994).
Chapter 6

Bibliography


Adams, A and Lindahl, T, (1975) Epstein-Barr virus genomes with properties of circular DNA molecules in carrier cells PNAS USA 72 1477-1481

Adldinger, HK, Delius, H, Freese, UK, Clarke, J, and Bornkamm, GW, (1985) A putative transforming gene of Jijoye virus differs from that of Epstein-Barr virus prototypes Virology 141 21-234

Ahearn, JM, Hayward, SD, Hickey, JC, and Fearon, DT, (1988) Epstein-Barr virus (EBV) infection of murine L cells expressing recombinant human EBV/C3d receptor PNAS USA 85 9307-9311


Alkhatib, G, and Bredes, DJ, (1988) High level eucaryotic in vitro expression of biologically active measles virus hemagglutinin by using an adenovirus type 5 helper-free vector system J Virol 62 2718-2727


Ambinder, RF, Shah, WA, Rawlins, DR, Hayward, GS, and Hayward, SD, (1991) Definition of the sequence requirements for the binding of the EBNA1 protein to its palindromic target sites in Epstein-Barr virus DNA J Virol 64 2369-2379


238


Austin, PJ, Flemmington, E, Yandava, CN, Stromunger, JL, and Speck, SH, (1988) Complex transcription of the BamH1 fragment H rightward open reading frame 1 (BHRF1) in latently and lytically infected B lymphocytes PNAS USA 85 3678-3682

Baer, R, Bankier, AT, and Buggin, MD, (1984) DNA sequence and expression of the B95-8 Epstein-Barr virus genome Nature 310 207-211


Barbas, CF III, (1993) Recent advances in phage display Curr Opin Biotech 4 526-530

Barbas, CF III, Björling, E, Chiodi, F, Dunlop, N, Cababa, D, Jones, TM, Zebedee, SL, Persson, MAA, Nara, PL, Norrby, E, and Burton, DR, (1992a) Recombinant human Fab fragments neutralise human type 1 immunodeficiency virus in vitro PNAS USA 89 9339-9343

Barbas, CF III, Crowe Jr, JE, Cababa, D, Jones, TM, Zebedee, SL, Murphy, BR, Chanock, RM, and Burton, DR, (1992b) Human monoclonal Fab fragments derived from a combinatorial library bind to respiratory syncytial virus F glycoprotein and neutralise infectivity PNAS USA 89 10164-10168


Bayer, ME, (1968) Areas of adhesion between wall and membrane of Escherichia coli J Gen Microbiol 53, 395-404


a repeated amino acid sequence of a *Plasmodium falciparum* antigen, Pf155, react with the native protein and inhibit merzoe invasion PNAS USA 83 2677-2681


Burnbom, HC, and Doly, J, (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA Nucleic Acids Res 7 1513


Chen, MR, Hsu, TY, Lin, SW, Chen, JY, and Yang, CS, (1991) Cloning and characterisation of cDNA clones corresponding to transcripts from the BamH1 G region of the Epstein-Barr virus genome and expression of BGLF2 J Gen Virol 72 3047-3055


Crawford, DH and Ando, I, (1986) EB virus induction is associated with B-cell maturation. Immunology 59 405-409


Cull, MG, Miller, JF, and Shatz, PJ, (1992) Screening for receptor ligands using large libraries of peptides linked to the C terminus of the lac repressor. PNAS USA 89(5) 1865-1869

244


Devlin, JJ, Panganiban, LC, and Devlin, PE, (1990) Random peptide libraries A
source of specific protein binding molecules Science 249(4967) 404-406

Diala, ES and Hoffman, RM, (1983) Epstein-Barr HR-1 virion DNA is very highly
methylated J Virol 45 482-483

di Guan, C, Li, P and Inonye, H, (1987) Vectors that facilitate the expression and
oufication of foreign peptides in Escherichia coli by fusion to maltose-binding protein
Gene 67 21-30

Dillner, J, Sternas, L, Kallin, B, Alexander, A, Ehlm-Henrikson, B, Jornvall, H,
Epstein-Barr virus-determined nuclear antigen PNAS USA 81(15) 4652-4656

Research 50 65-157 Academic Press Inc

Dopatka, HD and Schuy, W, (1996) Compact Epstein-Barr virus diagnosis based on a
defined antigen mix and specific IgA Res Virol 147 53-66


Virol 66 1931-1940

characterisation, distribution and strain differences J Virol 39 172-184

of a murine fascin homolog from mouse brain J Biol Chem 270(18) 10764-10770

purified heterophile antigen for the rapid diagnosis of infectious mononucleosis
compared with Epstein-Barr virus-specific serology Clinical and Diagnostic Virology 7
17-21

of a defective adenovirus expressing the pseudorabies virus glycoprotein gp50 and it's
use as a live vaccine J Gen Virol 71 2425-2431

Emmi, EA, Luka, J, Armstrong, ME, Keller, PM, Ellis, RW and Pearson, GR,
(1987) Identification of an Epstein-Barr virus glycoprotein which is antigenically
homologous to the varicella-zoster virus glycoprotein II and the herpes simplex virus
glycoprotein gB Virology 157 552-555

Epstein, MA, Achong, BG, and Barr, YM, (1964) Virus particles in cultured
lymphoblasts from Burkitt's lymphoma Lancet 1 702-703

Epstein MA, (1976) Epstein-Barr virus - is it time to develop a vaccine program? JNCI
56 607-700


249


Grossman, SR, Johannsen, E, Tong, X, Yalamanchili, R, and Kieff, E, (1994) The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the Jk recombination signal binding protein PNAS USA 91 7568-7572


Hammerschmidt, W, Sugden, B, and Baichwal, VR, (1989) The transforming domain of the latent membrane protein of the Epstein-Barr virus is toxic to cells when expressed at high levels J Virol 63 2469

Hammerschmidt, W and Sugden, B, (1988) Identification and characterisation of orILyt, a lytic origin of replication of Epstein-Barr virus Cell 5 427-433
Harris, A., Young, BD., and Griffin, BE., (1985) Random association of Epstein-Barr virus with host cell metaphase chromosomes in Burkitt’s lymphoma-derived cell lines J Virol 56 328-332


Henle, G., Henle, W., and Diehl, V., (1968) Relation of Burkitt’s tumour-associated herpes-type virus to infectious mononucleosis PNAS USA 59 94-101

Henle, G. and Henle, W., (1970) Observations on childhood infections with the Epstein-Barr virus J Infect Dis 121(3) 303-310


Henle, G. and Henle, W., (1976) Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma Int J Cancer 17 1-7

Henle, G. and Henle, W., (1979) The virus as the etiologic agent of infectious mononucleosis, 297-307 In Epstein, MA and Achong, BG., (eds),The Epstein-Barr virus Springer Verlag, Berlin

252


Longnecker, R and Kieff, E, (1990) A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and co-localises with LMP1 J Virol 64 2319-2326


Luka, J, Kallin, B, and Klein, G, (1979) Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by n-butryrate Virology 94 (1) 228-231


Lung, ML, Chan, KH, Lam, WP, Kou, SK, Choy, D, Chan, CW and Ng, MH, (1989) In situ detection of Epstein-Barr virus markers in nasopharyngeal carcinoma patients Oncology 46 310-317


Masucci, MG and Ernberg, I, (1994) Epstein-Barr virus Adaptation to life within the immune system Trends Micobiol 2 125-130


Matthews, DJ and Wells, JA, (1993) Substrate phage selection of protease substrates by monovalent phage display Science 260(5111) 1113-1117


Miller, G, Shope, T, and Coope, D, (1972) Epstein-Barr virus transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leucocytes PNAS USA 69 383-385

Miller, G, (1971) Human lymphoblastoid cell lines and Epstein-Barr virus (a review of their interrelationships and their relevance to the etiology of leukoproliferative states in man) Yale J Biol Med 43 358-384


262
Moore, KW, Vieira, P, Fiorentino, DF, Trounstine, ML, Khan, TA and Mosmann, TR, (1990) Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRF1 Science 248 1230-1234


Morrow, RH Jr, (1985) Epidemiological evidence for the role of falciparum malana in the pathogenesis of Burkitt’s lymphoma In Lenoir, G, O’Conor, G and Olweny,
CLM, (eds), Burkitt’s Lymphoma A Human Cancer Model (IARC Scientific Publications No 60), Lyon, IARC, 177-186


Nemerow, GR, Mold, C, Schwend, VK, Tollefson, V, and Cooper, NR, (1987) Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor at B cells sequence homology of gp350 and C3 complement fragment C3d J Virol 61 1416-1420


265


Patarroyo, M, Blazar, B, Pearson, G, Klein, E, and Klein, G, (1980) Induction of the Epstein-Barr virus cycle in B-lymphocyte-derived lines is accompanied by increased natural killer (NK) sensitivity and the expression of EBV-related antigen(s) detected by ADCC reaction Int J Cancer 26(3) 365-371

Paterson, Y, Englander, SW, and Roder, H, (1990) An antibody binding site on cytochrome c defined by hydrogen exchange and two-dimensional NMR Science 249(4970) 755-759


Pellett, PE, Biggin, MD, Barrell, B, and Roizman, B, (1985) EBV genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus1 J Virol 56 807-813

Pestring, RJ, Zellmer, RB, Sulak, LE, Banks, PM, and Clare, N, (1991) Hodgkin’s disease in association with human immunodeficiency virus infection pathologic and immunologic features Cancer 67(7) 1865-1873


Purtilo, DT, (1991) X-linked lymphoproliferative disease (XLP) as a model of Epstein-Barr virus-induced immunopathology Semin Immunopathol 13 181-197

Purtilo, DT and Hinnchs, S Detection of Epstein-Barr virus induced diseases by laboratory techniques INCSTAR Corporation

Qualtiere, LF, Chase, R, and Pearson, GR, (1982) Identification of Epstein-Barr virus strain differences with monoclonal antibodies to a membrane glycoprotein PNAS USA 79 616-620


Rawlins, DR, Milman, C, Hayward, SD, and Hayward, GS, (1985) Sequence-specific DNA binding of the Epstein-Barr nuclear antigen 1 (EBNA1) to clustered site in the plasmid maintenance region Cell 42 859-868

268


Reisman, D and Sugden, B, (1986) Trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1 Mol Cell Biol 6 3838-3846


Rowe, M, Young, LS, Cadwallader, K, Pettu, L, Kieff, E, and Rickinson, AB, (1989) Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA3 family of nuclear proteins J Virol 63 1031-1039


third promoter, distinct from the promoters used in latently infected lymphocytes PNAS USA 88 6550-6554.


Scott, JK and Smith, GP, (1990) Searching for peptide ligands with an epitope library Science 249 386-390


Seibl, R and Wolf, H, (1985) Mapping of Epstein-Barr virus proteins on the genome by translation of hybrid selected RNA from induced P3HR1 cells and induced Raji cells Virology 141 1-13


Shims, SL, and Sculley, TB, (1994) Modulation of vimentin, the CD40 activation antigen and Burkitt’s lymphoma antigen (CD77) by the Epstein-Barr virus nuclear antigen 4 Virology 202 16-24


Skare, J. and Strominger, JL., (1980). Cloning and mapping of Bam HI endonuclease fragments of DNA from the transformîng B95-8 strain of Epstein-Barr virus. PNAS USA 77: 3860-3864.


Svedmyr, E. and Jondal, M., (1975). Cytotoxic effector cells specific for B cell lines transformed by Epstein-Barr virus are present in patients with infectious mononucleosis. PNAS USA 72: 19622-1626.

immunologic, and clinical observations on a patient during the incubation, acute, and convalescent phases of infectious mononucleosis Clin Immunol Immunopathol 30 437-450

Swimmer, C, Lehar, SM, McCafferty, J, Chiswell, DJ, Blattler, WA, and Guild, BC, (1992) Phage display of ricin B chain and it’s single binding domains system for screening galactose-binding mutants PNAS USA 89 3756-3760


275
Thorley-Lawson, DA, and Geilinger, K., (1980) Monoclonal antibodies against the major glycoprotein (gp350/220) of Epstein-Barr virus neutralise infectivity PNAS USA 77 5307-5311


Van der Weid, T and Langhorne, J, (1993) The role of cytokines produced in the immune response to the erythrocytic stages of mouse malarias Immunobiology 189 397-418


restrictions associated to the mechanism of immune recognition J Mol Biol 254(3) 497-504


Walls, D and Perricaudet, M, (1991) Novel downstream elements upregulate transcription from an Epstein-Barr virus latent promoter EMBO J 10 143-151


Wang, F, Gregory, CD, Sample, C, Rowe, M, Liebowitz, D, Murray, R, Rickinson, AB, and Kieff, E, (1990) Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes EBNA-2 and LMP1 co-operatively induce CD23 J Virol 64 2309-2318


Zhang, Q, Gutsch, D, and Kenney, S, (1994a) Functional and physical interaction between p53 and BZLF1 Implications for Epstein-Barr virus latency Mol Cell Biol 14 1929-1938


Zhang, Q, Holley-Guthne, E, Ge, J-Q, Dorsky, D, and Kenney, S, (1997) The Epstein-Barr virus (EBV) DNA polymerase accessory protein, BMRF1, activates the essential downstream component of the EBV oriLyt Virology 230 22-34


Zheng, YM, Tuppin, P, Hubert, A, Jeannel, D, Pan, YJ, Zeng, Y and de Thé, G, (1994a) Environmental and dietary risk factors for nasopharyngeal carcinoma A case-control study in Zangwu County, Guangxi, China Br J Cancer 69 508-514


280
Appendix A
I Solutions for DNA work

i General solutions

0.5 M EDTA
186 g EDTA
800 ml distilled water
6 g NaOH pellets
pH to 8.0 with 5 M NaOH
Adjusted volume to 1 L with water

50X TAE
242 g Tris
57 ml Acetic acid
100 ml 0.5 M EDTA pH 8.0
Adjusted to 1L with distilled water

5X TBE
54 g Tris
27.5 g Boric acid
20 ml 0.5 M EDTA pH 8.0
Adjusted to 1L with distilled water

Ethidium bromide (10mg/ml)
0.1 g Ethidium bromide
10 ml Distilled water
Stored in dark

DNase-free RNAse (1 mg/ml)
1 mg RNAse A
1 ml Distilled water
100°C, 30 min
Cool slowly, store -20°C
Agarose gel loading dye
400 mg Sucrose
2.5 mg Bromphenol blue
1 ml Distilled water

TE buffer
10 mM Tris
1 mM EDTA pH 8.0

**ii Solution for mini-preparation and maxi-preparation of plasmid DNA**

Solution I
50 mM Glucose
25 mM Tris Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Solution II
0.2 N NaOH (freshly diluted from a 10N stock)
1% SDS

Solution III
60 ml 5M Potassium acetate
11.5 ml Glacial acetic acid
28.5 ml Distilled water

**II Media and solutions for Thiofusion kit.**

Ampicillin (100 mg/ml)
1 g Ampicillin
10 ml Distilled water

Filter sterilised, aliquoted in 1 ml aliquots, stored at -20°C
10X M9 salts (per L)

60 g Na₂HPO₄
30 g KH₂PO₄
5 g NaCl
10 g NH₄Cl
900 ml distilled water

pH 7.4 with 10 M NaOH

Autoclaved and stored at room temperature

RM medium (per L)

20 g Casamino acids
10 ml 100% Glycerol
880 ml Distilled water

Autoclaved and added the following

100 ml 10X M9 salts
1 ml 1 M MgCl₂
1 ml Ampicillin (100 mg/ml)

Mixed and stored at 4°C

RMG-amp media (per L)

20 g Casamino acids
15 g Agar technical
875 ml Distilled water

Autoclaved and cooled to ~55°C, added the following

100 ml 10X M9 salts
1 ml 1M MgCl₂
10 ml 50% Glucose
1 ml Ampicillin (100 mg/ml)

Plates stored at 4°C
Induction medium (per L)
2 g Casamino acids
885 ml Distilled water
Autoclaved and added the following
100 ml 10X M9 salts
1 ml 1M MgCl$_2$
10 ml 50% Glucose
1 ml Ampicillin (100 mg/ml)

LB medium (per L)
10 g Bacto-tryptone
5 g Yeast extract
5 g NaCl
Autoclaved and stored at room temperature

LB agar
10 g Tryptone
5 g Yeast extract
5 g NaCl
15 g Agar technical
Autoclaved and plates stored at 4°C

SOB medium (per L)
20 g Tryptone
5 g Yeast extract
0.5 g NaCl
10 ml KCl (250 mM)
Adjusted pH to 7.0 with 5 M NaOH
Autoclaved, cooled to ~5°C and added
10 ml 1 M MgCl$_2$
Stored at room temperature
SOC medium (per L)
1 L SOB
7.5 ml 50% Glucose (filter sterilised)
Stored at room temperature

Osmotic Shock Solution # 1
20 mM Tris-HCl, pH 8
2.5 mM EDTA
Autoclaved,
20% Sucrose (Filter sterilised)
Stored at room temperature

Osmotic Shock Solution # 2
20 mM Tris-HCl, pH 8
2.5 mM EDTA
Autoclaved and stored at room temperature

III Solutions for SDS PAGE
1 M Tris Cl pH 6.8
15 M Tris Cl pH 8.8
10% SDS
10% Ammonium persulphate (APS)
Acrylagel
Bis-acrylagel
TEMED
1 M Dithiothreitol

10X Running buffer (500 ml)
15 138 g Tris
71 125 g Glycine
5 0 g SDS
To 500 ml with distilled water
2X Sample buffer
0.8 ml 1M Tris Cl pH 6.8
0.4 g SDS
0.02 g Bromophenol blue
2 ml Glycerol
To 8 ml with sterile distilled water
Add ¼ volume of 1 M dithothreitol to sample buffer, then dilute ½ with distilled water before adding an equal volume to the protein sample.

Destain (per L)
100 ml Acetic acid
400 ml Methanol
500 ml Distilled water

Coomassie blue stain (per 200 ml)
1 g coomassie blue R
200 ml destain

IV Solutions for Western Blotting
10X Tris Glycine buffer (per 500 ml)
14.5 g Glycine
29 g Tris
1.85 g SDS
Made up to 1 L with distilled water, adjusted to pH 8.3

Transfer Buffer (per L)
100 ml 10X Tris Glycine (pH 8.3)
700 ml Distilled water
200 ml Methanol
TBS (1X /L)
6 1 g  Tris
8 8 g  NaCl
Made up to 1 L with distilled water and adjusted to pH 7.5 with HCl
Autoclaved and stored at room temperature

TBST (0.1%)
1 L  TBS (as above)
1 ml  Tween 20

Blotto
50 ml  TBS (as above)
25 μl  0.05% Tween 20 (0.5 ml/L)
2 g  5% non-fat dry milk 50 g/L (Marvel)
0.5 g  NaN₃

V Solutions for protein purification with PAO resin
1 M Phosphate buffer
136.1 g  KH₂PO₄
174.2 g  K₂HPO₄
156.0 g  NaH₂PO₄
142.0 g  Na₃HPO₄ (anhydrous)

β-mercaptoethanol, 14.4 M stock

Running buffer
100 mM  Phosphate buffer
20 mM  NaCl
0.1 mM  EDTA
Leupeptin (2 mg/ml)
20 mg Leupeptin
10 ml Sterile distilled water
Stored at -20°C

PMSF (17.4 mg/ml)
174 mg PMSF
10 ml Isopropanol
Stored at -20°C

VI Solutions for ELISA with recombinant EBV antigens
Coating buffer (per L)
4.24 g Na₂CO₃
5.4 g NaH₂CO₃
Made up to 1 L with distilled water and adjusted to pH 9.6

TBS, TBST and Blotto - As for Western blotting

Diluent for sera
1X TBS
5 % Foetal calf serum (FCS)
0.05 % NaN₃
0.05 % Tween

VII Media and solutions for peptide libraries (NEB)
LB/IPTG/Xgal agar (per L)
1 L LB medium (see section II)
15 g Agar technical
Autoclaved and cooled to <70°C
Agarose top (per L)
10 g Tryptone
5 g Yeast extract
5 g NaCl
1 g MgCl₂ 6H₂O
7 g agar technical

Autoclaved and stored solid at room temperature in 50 ml aliquots

Minimal media (per L)
100 ml 10X M9 salts (as for Thiofusion kit)
15 g Agar technical
8 ml 50% Glucose
2 ml 1 M MgSO₄
1 ml 100 mM CaCl₂
1 ml Thiamine (10 mg/ml)

All components are autoclaved separately and cooled to < 70°C (glucose and thiamine are filter sterilised) before combining

Blocking buffer
0.1 M NaHCO₃ (pH 8.6)
5 mg/ml BSA
0.02% NaN₃

Filter sterilised and stored at 4°C

TBS (as for Western blotting)

PEG/NaCl
20% (w/v) Polyethylene glycol-8000
2.5 M NaCl

Autoclaved and stored at room temperature
Iodide buffer
10 mM Tris Cl (pH 8.0)
1 mM EDTA
4 M NaI
Stored at room temperature

IPTG/Xgal
1.25 g IPTG (isopropyl β-D-thiogalactoside)
1 g Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)
25 ml Dimethyl formamide
Stored at -20°C in the dark

Elution buffer
0.2 M Glycine (pH 2.2 with HCl)
1 mg/ml BSA

Neutralising solution
1 M Tris-HCl (pH 9.1)

VIII Media and Reagents for 15mer library

Blocking solution (per ml)
1 ml 0.1 M NaHCO₃
5 mg Dialysed BSA
0.1 μg Streptavidin
4 μl NaN₃ (5% stock)

Kanamycin
100 mg Kanamycin
1 ml Distilled water
Filter sterilised and stored at 4°C
Sodium azide (5%)  
50 mg NaN₃  
950 µl Distilled water

TB soft agar (100 ml)  
1 g Tryptone  
0.5 g NaCl  
0.75 g Agar technical  
100 ml Distilled water  
Autoclaved and stored at room temperature

TBS (as per Western blotting)  

TBS/gelatin  
0.1 g Gelatin  
100 ml TBS  
Autoclaved and stored at room temperature

TBS/Tween (0.5%)  
200 ml TBS  
1 ml Tween 20

Tetracycline  
100 mg Tetracycline  
5 ml Ethanol  
Filter-sterilised and stored at -20°C

Potassium phosphate buffer (per 100 ml)  
23.1 g KH₂PO₄ (anhydrous)  
12.54 g K₂HPO₄ (anhydrous)  
Made up to 100 ml with distilled water and autoclaved
Terrific broth (per L)
12 g Tryptone
24 g Yeast extract
4 ml Glycerol
Made up to 1L with distilled water and autoclaved in 90 ml portions
To each 90 ml portion 10ml autoclaved potassium phosphate buffer was added

NZY medium (per L)
10 g NZ amine A
5 g Yeast extract
5 g NaCl
Dissolved in 1 L distilled water, pH adjusted to 7.5 with NaOH, autoclaved and stored at room temperature

NZY agar - As above with 15 g agar technical

TNT (per L)
1.21 g 10 mM Tris pH 8.0
8.77 g 150 mM NaCl
0.5 ml 0.05% Tween 20

TBS and Blotto as for Western blotting

TNT A (per 100 ml)
100 ml TNT
0.1 g BSA

TNT B (per 100 ml)
100 ml TNT
0.1 g BSA
0.1 ml NP-40
Deglycosylation buffer
20 mM Na₂HPO₄
40 mM EDTA
Adjusted to pH 7.2

**IX Reagents for sequencing**

10X TBE (per 500 ml)
54 g Tris base
27.5g Boric acid
20 ml 0.5 M EDTA (pH 8.0)
Made up to 500 ml with ultra pure water
1X concentration was used for polyacrylamide gel preparation

10% Ammonium persulphate
0.1 g APS
1 ml Ultra pure H₂O
6% denaturing polyacrylamide gel was prepared for sequencing the Conley library phage and 8% for the NEB library phage

Va = volume of acrylamide
Vb = volume of bis-acrylamide
Vt = total volume of gel mix 150 ml
C = % crosslinking 5.2%
A = % gel 6/8%

\[
Va = \frac{Avt}{30} \quad Vb = \frac{ACt}{200}
\]

\[
Va = 6 \times 150 / 30 = 30 \text{ ml}
\]

\[
Vb = 6 \times 5.2 \times 150 / = 24 \text{ ml}
\]
6% Denaturing PAG

63 g Urea
30 ml Acrylamide
24 ml Bisacrylamide
15 ml 10X TBE

made up to 150 ml w/ ultra pure water

650 μl 10% APS and 150 μl TEMED were added directly before pouring

Developer (5 L)

1 50 L H₂O
1 25 L Concentrated developer solution
2 25 L H₂O

stir 2 min

Fixer (5 125 L)

3 625 L H₂O
1 250 L Concentrated fixer A
0 250 L Concentrated fixer B

stir 2 min